

Time for a firm grasp on UK science priorities

The Labour party is expected to win the election in the United Kingdom next week, but it has so far provided little to inspire researchers. The challenges that face it are chronic and require management with insight.

Unless the psephologists prove themselves to have been spectacularly inept, Britain will wake up on Friday next week (2 May) to its first change of government in 18 years. On the basis of what little has been said about science during the general election campaign, there are few overt reasons for researchers as such to be enthusiastic about the prospect of a Labour administration. Indeed, the promises of Tony Blair, the Labour leader, to place science "at the heart of government" have had little of the resonance — and raised little of the fervour — of the similar words that helped sweep Harold Wilson to power in 1964 on the back of a commitment to create a new Britain "forged in the white heat of this [technological] revolution".

Wilson's strategy failed, of course. A powerful Ministry of Technology turned out to be an unwieldy monster, while promises of increased support for science evaporated in the crises that gripped the economy towards the end of the decade. Seen in this light, the cautious style of electioneering adopted by today's Labour party might seem prudent. Moreover, some significant initiatives introduced by the Conservative government have proved partly beneficial: technology foresight and research assessment have both focused attention where it was needed within the UK science base, and have attracted respect and even emulation in other countries. Increased autonomy of science institutions has led to enhanced productivity.

But the indigenous technological industrial base of the United Kingdom that was, above all, supposed to be the beneficiary still lacks international competitiveness. Another major task lies within government itself: to forge a genuinely transdepartmental science policy. The failure to achieve this has led to additional burdens on the research councils due to government departments retreating from research

support, underlining the absence of an overall strategy.

Providing a better career framework for researchers is still a critical issue for any government that cares about science. An improved approach must retain the flexibility needed to ensure that resources are focused on the most productive fields of science, with the elements of continuity in support needed to persuade potential recruits that science offers a worthwhile and rewarding career. Too often over the past 18 years, the first of these dominated the second. Researchers who are intellectually and professionally mobile need a sense that they are valued for those very qualities.

Finally, Britain's next government, whatever its political complexion, will face growing pressure to increase the social accountability of the research community. Accommodating such pressure will itself require a more sophisticated strategy for enhancing the involvement and understanding of the public where science and the concerns of society connect or even collide. This task must be seen in terms of encouraging public involvement in technology assessment and technological choice.

The Wilsonian faith in technology and in government push proved naive and impractical economically. The Thatcherite zeal for a free market in ideas and people has proved powerful but inadequate. Finding an appropriate balance is the next government's principal task. But Labour's ideas still lack conviction, and its pre-election team lacks anyone with detailed insight into managing research. If elected, it will need the help of politically sympathetic scientists and technologists to turn its vague promises into concrete and effective actions. In turn, the sensitivity of such individuals to political realities will be critical to the government's success in managing British science. □

Tip of an iceberg?

All of Japan's semi-government research and development needs critical re-examination.

Japan is abuzz this week with calls for PNC, a semi-government organization (*tokushu hojin*) responsible for research and development in nuclear power, to be dissolved following a series of scandalous attempts by PNC officials to cover up mistakes made in accidents at its facilities (see page 746). But many of Japan's *tokushu hojin* have similar inherent defects.

Tokushu hojin, of which there are dozens consuming a large portion of the government's budget, are an odd breed of organization that span the public and private sector. Many are involved in government research and development and most of their funds come from the taxpayer. They are tightly linked to the government ministry or agency responsible for overseeing them. Bureaucrats often do a stint of management at a *tokushu hojin* in mid-career, while senior bureaucrats often retire to *tokushu hojin* to receive high salaries for a few years and a second retirement allowance.

Not surprisingly, ministries and agencies jealously guard their *tokushu hojin* and are reluctant to see them dissolved or reformed, no

matter how moribund they are. The Science and Technology Agency (STA), for example, has clearly been slow to act against PNC despite overwhelming evidence of incompetence and deception since the Monju accident. Two years ago, Makiko Tanaka tried to push for reform of *tokushu hojin* under the STA, which she then headed. But almost nothing happened (see *Nature* 373, 551; 1995).

PNC escaped unscathed then, but now seems destined for a major overhaul. But what of the many other *tokushu hojin*, such as the National Space Development Agency and the New Energy and Industrial Technology Development Organization?

Not all are necessarily bad. The Institute of Physical and Chemical Research (RIKEN), one of Japan's leading research organizations, for example, seems to draw strength from its semi-government status, which allows greater autonomy and flexibility than is found in ordinary government research institutes. But a thorough re-examination of all research-related *tokushu hojin* is in order at a time when Japan plans to pump much more money into government research. □



Austrian gene food petition puts pressure on European partners

[MUNICH] Austria has become the latest focus of European opposition to biotechnology. Twenty per cent of the population turned out last week to sign a petition calling for a ban on genetically modified food and on patents on genetically modified plants and animals.

The action increases the pressure on the Austrian government to maintain its opposition to efforts by the European Union (EU), which it joined relatively recently, to provide a more sympathetic legal environment for the biotechnology industry.

The national petition, known as a *Volksbegehren*, was organized by the Austrian ministry of internal affairs after its initiators — a group of non-governmental organizations (NGOs) led by the environmentalist groups Greenpeace Austria and Global 2000 — obtained the necessary support from eight members of parliament.

More than 1.2 million Austrians visited polling stations to sign the petition which made three demands: "No food from genetic laboratories in Austria", "No field trials of genetically manipulated crops in Austria" and "No patents on life".

The turnout was unexpectedly high — the second-highest in Austria's history of national

petitions, and the highest ever for a grass-roots initiative. This has forced a quick response from the government, which is an uneasy coalition of Austria's two main parties, the centre-left Social Democrats and the centre-right Austrian Popular Party.

Chancellor Victor Klima has called a summit meeting for next Monday, 28 April. Together with five cabinet ministers, he will discuss with representatives of the NGOs their list of 40 specific demands. These include toughening the approval procedures for genetic experiments and increasing the power of the ethical committees involved in the procedures. They also want the manufacturers of gene-manipulated products to have explicit liability if their products cause environmental damage or health problems.

The NGOs want a ban on the local production of foods containing genetically modified ingredients — a ban on the import of such foods is considered unenforceable — and a five-year moratorium on field trials of genetically modified crops.

The government's response to these demands could significantly influence EU policies on genetic engineering, particularly if it accepts a significant number of them. In

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Food for thought: a selection of foodstuffs that use soya as a constituent ingredient.

early February, Austria imposed a temporary ban on imports of Ciba's genetically engineered maize, even though it had been licensed for import into the EU last year.

Under a clause in the EU's directive on the deliberate release of genetically manipulated organisms, a three-month ban is allowed if a member state can provide evidence of risk to the environment or health.

Austria argued that the crop presented a risk because it contains a marker gene conferring antibiotic resistance which it says could, in principle, be transferred to humans or animals. Luxembourg has since imposed a similar ban, and Italy has banned the growing of crops from the maize seeds.

A European-level committee is considering the Austrian claims and is due to deliver its verdict by 15 May, when the temporary ban expires. But as the Austrian report apparently cites no new evidence, the European Commission is likely to rule against Austria's appeal.

This could bring to a head simmering tensions in Europe over genetically engineered foods. Only two weeks ago, the European Parliament, fired by memories of the commission's alleged mishandling of bovine spongiform encephalopathy, chastised the commission for its recent decision to exclude genetically engineered seeds that have already been licensed — such as Ciba's maize — from new, interim rules requiring them to be labelled (see *Nature* 386, 532; 1997).

If Austria takes a hard line on genetic engineering, this could also influence the fate of the commission's draft directive on the legal protection of genetically engineered products. A previous attempt by the commission to win the support of the European Parliament for such a directive failed two years ago, partly because of ethical concerns. The new directive is scheduled to receive its first parliamentary hearing next month.

In Austria, environmentalists are claiming that the petition represents a significant victory. "The vote gives a clear signal to the government that the population does not want genetically engineered food," says Matthias Schickhofer of Greenpeace Austria. **Alison Abbott**

US advisory panel backs ITER design

[WASHINGTON] A US Department of Energy (DoE) advisory panel has endorsed the design of the International Thermonuclear Experimental Reactor (ITER), even though it acknowledges that the reactor may not achieve one of its original design goals, plasma ignition.

In a report last week to Martha Krebs, the director of energy research, the Fusion Energy Sciences Advisory Committee said it found "no insurmountable obstacles" to prevent the proposed \$10-billion reactor from achieving its objectives.

The panel said that the design represents a "sound basis" for the United States to continue its relatively small planned contribution to ITER's construction phase. But its report acknowledged that the machine may be unable to demonstrate controlled ignition and extended burn, one of the original objectives set by the four ITER parties in 1992.

Krebs acknowledged that the question surrounding the demonstration of controlled ignition "is the sensitive result from the study", pointing out that no-one disputes that the project represents "an important set of science experiments". She added: "The

question is how will Congress receive that."

The House of Representatives Science Committee last week approved authorizing legislation to add \$15 million to DoE's \$225-million request for fusion energy research in fiscal year 1998, after several years in which Congress has cut the fusion budget. The Appropriations Committee has yet to act on the budget request.

The Science Committee has asked for a report on the appropriate role of the US fusion community in ITER once the design phase is completed next year. It points out that there is no official indication from the ITER project group, nor from participating parties, on how they intend to proceed to the construction phase.

Robert Conn, dean of engineering at the University of California at San Diego, and chair of the ITER review panel, said fusion researchers would be "ecstatic" with the machine, whether it achieves ignition or not.

Asked by Krebs whether he believed that the design for ITER is worth the proposed annual US contribution of \$50 million, Conn responded, "at \$50 million it's a terrific bargain". He added: "We're getting off on the cheap". **David Kramer**

Scientists dispute wisdom of Canada reopening fishery

[MONTREAL] Canadian scientists are divided over the government's announcement last week that it is partially to reopen the east coast cod fishery, closed in 1992 because of declining stocks.

Some are openly critical of the decision. Ransom Myers, professor of ocean studies at Dalhousie University in Halifax, Nova Scotia, says stocks remain at a very low level. Myers, who until recently was employed by the federal department of fisheries and oceans, argues that the fisheries minister was not sufficiently informed of the risks to long-term fishing prospects in the region. Jeffery Hutchings, also of Dalhousie, describes the move as "a management decision inconsistent with the best stock analysis that scientists put forth".

But William Doubleday, director-general of science for the fisheries department, says that "there are considerable uncertainties about abundances", and that scientists disagree about the risks of reopening the fishery. He agrees that stocks remain at low levels and that the scientific data have remained unchanged since last June, however. He denies the decision is related to the timing of the forthcoming federal elections.

The reopening of the fishery will allow 10,000 tonnes of cod to be taken off the southern coast of Newfoundland, compared to about 35,000 tonnes when it was closed in 1992. In the northern Gulf of St Lawrence, 6,000 tonnes will be allowed, rather than the former 40,000. And the sentinel fishery, at 2,000 tonnes, will be allowed to monitor changes in the southern gulf.

The government's move is based on recommendations of the Fisheries Conservation Resource Council, an independent body of fishing industry representatives, scientists and other interested parties. According to Doubleday, the main reason for limited reopening is to obtain information to provide a better basis for a long-term decision.

The new limits are quite low compared to historic levels — about one-third of the catch taken when the fishery was closed, and less than a quarter of what it was in previous years. "So the scientific view is that this doesn't pose any significant conservation risk to the stocks and it's an opportunity to get better information about them," says Doubleday.

Extensive discussions took place with fishermen about how they would catch their quota responsibly, from the point of view of conservation.

The new quotas are for this year only, says Doubleday. The stock will be assessed next January, taking into account a new scientific survey, a further sentinel review and the results of this year's fishing. **David Spurgeon**

Accident fall-out threatens Japan's nuclear company

[TOKYO] Japan's Power Reactor and Nuclear Fuel Development Corporation (PNC) is threatened with drastic reform — and perhaps even dissolution — after a series of accidents and cover-ups at nuclear facilities. PNC is a huge semi-public organization responsible for a large portion of Japan's research and development on nuclear power.

The Science and Technology Agency (STA), which oversees PNC, would be greatly affected by such a move, as more than 40 per cent of its annual budget goes to support the development of nuclear power, much of it — ¥160 billion (US\$1.4 billion) — to PNC.

Calls for reform from the top levels of Japanese government came after the revelation this month that PNC officials had falsified reports about a fire and explosion in March at a facility used for the 'bitumenization' of low-level radioactive waste in Tokai, northeast of Tokyo (see *Nature* 386, 209; 1997).

Responding to pressure from the police, the STA has filed a criminal complaint against the organization, as well as against PNC officials who tried to cover up a false report to the agency claiming that the fire had been extinguished shortly after it started. In fact, the waste facility blew up ten hours after the fire was reported to have been extinguished.

Three days before the STA filed its complaint, a minor leak of heavy water and tritium gas occurred at another PNC facility, the Fugen advanced thermal reactor in Fukui Prefecture on the Japan Sea coast. PNC took 30 hours to report the leak to the agency and local government. It has since been revealed that similar leaks have occurred on 18 previous occasions since 1992. But none was reported by PNC, despite an agreement with the local government to do so.

The STA has accepted that no laws were broken at Fugen. But the agency has shut down the reactor to make its operators aware of what Kaname Ikeda, director-general of STA's nuclear safety bureau, calls their "moral and social" obligation to report leaks.

The Fugen and Tokai incidents follow a leak of sodium coolant at PNC's Monju fast-breeder reactor in December 1995. PNC officials attempted to hide a videotape recorded shortly after that leak (see *Nature* 379, 196; 1996). With three of its key facilities closed down, PNC is crippled.

Japan's prime minister, Ryutaro Hashimoto, has been repeatedly questioned by the media about PNC, and is exasperated by its failings. Seiroku Kajiyama, chief cabinet secretary, has called for a review of PNC with "nothing off limits", including its "dissolution if that is what is needed".

Last Friday (18 April), the STA hurriedly

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Covering up: An official puts on protective gear to tackle the accident at the Tokai nuclear plant.

convened a PNC reform committee of nine academics and industrialists headed by Horiyuki Yoshikawa, former president of Tokyo University. Ikeda says the committee has been told by Riichiro Chikaoka, the cabinet member who heads the STA, to consider "all options". These include privatization, partial privatization, reorganization under the government and dissolution.

But the private sector has expressed little interest in taking over PNC, apart from its demonstration nuclear fuel enrichment facility in Ningyo-Toge in Okayama Prefecture and its fuel reprocessing and fabrication facilities in Tokai, which are already run on a commercial basis.

Hashimoto is said to be sceptical about total privatization and believes that the research and development components of the organization should remain in the government domain. One possibility is that PNC will be carved up, with part of its responsibilities being transferred to the Atomic Energy Research Institute, also overseen by the STA and located in Tokai.

Ikeda expects the committee to reach a conclusion in only a few months because the reform of PNC is likely to become part of the government's much bigger proposed administrative reform of ministries and agencies. That reform is expected to be formulated by the end of September, when there will be an election for leadership of the ruling Liberal Democratic party.

Although the STA has been rocked by the PNC scandal, some observers think it could have positive ramifications for other research supported by the agency. It could drive the STA to divert more funds to non-nuclear research, such as the life sciences. This trend is already occurring with the agency's recent large investments in brain research and limited increases in the budget for nuclear power (see *Nature* 383, 7; 1995). **David Swinbanks**

Baylor backed over 'falsified data' claims

[SAN DIEGO] The US Office of Research Integrity (ORI) has concluded that a neuroscientist formerly at Baylor College of Medicine in Houston, Texas, intentionally falsified research data in five published articles and grant applications over a five-year period.

The case has taken on national significance because of the potential implications of a lawsuit which the researcher, Kimon Angelides, has filed against Baylor and those of its scientists and administrators who were originally involved in accusing him of misconduct (see *Nature* 383, 107; 1996 & 384, 105; 1997).

The outcome of the suit is being closely watched both by the ORI and the Association of American Medical Colleges. The lawsuit, which Angelides filed in 1995 for wrongful termination, defamation and other alleged offences, has attracted this attention because of its potential to derail the process by which universities conduct scientific misconduct investigations.

According to an ORI report that was released last month, Angelides should be prohibited from receiving federal grants for five years because of "a lack of honesty and integrity". Angelides now works at the University of Durham in England, having been fired by Baylor in 1995.

The ORI has ordered Angelides to notify five journals of the falsifications as a condition of receiving any future government grants. This is expected to trigger the retraction of the articles, which were published with co-authors from Yale University and the University of Michigan. No co-author was implicated by ORI in scientific misconduct.

Angelides has appealed against ORI's findings to the Departmental Appeals Board. This is setting up a three-person panel which will determine the federal government's final word on the case's administrative aspects.

In the appeal, Angelides' attorney wrote that the researcher "disputes each finding in which he is accused of falsification, fabrication and [of being] the sole perpetrator of scientific misconduct. To the extent that any errors were made in grant applications or scientific papers, such were not the result of any intent to deceive".

The attorney, James V. Pianelli, also asked the appeals board to delay its hearing until the conclusion of the lawsuit that Angelides has filed. The board's panel is to consider that request soon.

In his lawsuit, Angelides also contends that he should be able to sue Baylor in Texas state court. But Baylor believes it is immune from lawsuits such as that filed by Angelides, as it was following federal requirements to investigate misconduct allegations. The medical college and its allies also argue that

such lawsuits in state court will have a chilling effect on professorial participation in scientific misconduct probes.

Baylor is appealing in federal court, with oral arguments set for the first week in June. A decision by the US Court of Appeals for the Fifth Circuit in New Orleans is expected to follow between 30 and 60 days later.

An attorney for Baylor, A. John Harper II, declines to comment on the substance of the ORI report. But he says that Baylor was "pleased that its findings in the Angelides matter have been upheld by ORI".

In parallel with the administrative and civil battles, Angelides remains under criminal scrutiny by the US Attorney's Office in Houston, which started an inquiry late last year into possible fraud charges for alleged false reporting on grants Angelides received from the National Institutes of Health (NIH).

A decision on whether criminal charges will be filed is not expected for some time because of an agreement said to have been made between federal prosecutors and Angelides. The agreement allows prosecutors to extend the statute of limitations for a criminal charge while Angelides is out of the country. It may allow prosecutors to observe the hearing of the appeals board, seeking information to support a criminal indictment.

All of these developments are being observed by officials at the University of Durham, who are declining to comment

until Angelides' appeals are complete.

At Baylor, Angelides' laboratory examined sodium channels in nerve cells in a search for medications or drug delivery routes. It was on NIH grant applications for such research from 1988 to 1992 that he is alleged by the ORI to have repeatedly falsified and/or fabricated results.

The five publications in question involved his sodium-channel exploration with laboratory-engineered antibodies, in particular an antibody created in his Baylor laboratory. These experiments produced figures and supporting text which were provided to the co-authors at Yale for use in their articles.

Yale officials say that they are waiting for official notification of the ORI findings. They are expected to retract the articles. The Michigan co-author is a former doctoral student at Baylor whose work is said by ORI to have been manipulated by Angelides.

After reviewing Baylor's report, ORI ruled that Angelides manipulated experimental results to produce desired figures for publication, mislabelled the type of nerve cells (central versus peripheral rat nerve tissue) used in experiments, or falsified the molecular weight of specimens.

The articles appeared in the *Proceedings of the Royal Society of London*, the *Proceedings of the National Academy of Science*, *Glia*, *Brain Research* and the *Annals of the New York Academy of Science*.

Rex Dalton

Patent office replies to fears over ESTs

[WASHINGTON] The head of the US Patent and Trademark Office has replied to criticism from the National Institutes of Health (NIH) about the office's decision to allow the patenting of short DNA sequences known as expressed sequence tags (ESTs) on the basis of their utility as molecular probes.

The NIH, led by its director, Harold Varmus, has predicted that this policy could quash research and product development because rights to an EST could encompass rights to the full-length gene to which it corresponds (see *Nature* 386, 312; 1997). But Bruce Lehman, the Commissioner of Patents and Trademarks, has responded that broad patent claims will not result from the policy.

"Patent claims, limited in scope to a specific novel and non-obvious EST, generally should not preclude the future patenting of the corresponding, later discovered, full-length gene of known function" or of therapeutic technologies arising from it, Lehman wrote.

Varmus had written to Lehman last month that he was "deeply concerned" about the EST announcement. Varmus pointed out that the NIH had decided in

1994 that "it was not in the best interest of the public health or science to pursue patents on partial or full gene sequences for which function and practical utility are unknown".

In his reply, Lehman also stressed that usefulness as a probe alone would not qualify an EST as patentable. "Mere allegation of the utility of an EST as a probe without further disclosure is not sufficient to meet the utility and enablement criteria," Lehman wrote.

Some claim that the response represents a retreat from the patent office's earlier policy announcement, because Lehman is acknowledging that an inventor must have some knowledge of the function or utility of the target gene for an EST used as a probe to satisfy legal requirements for patenting.

But Lawrence Goffney, deputy commissioner of patents, who made the initial announcement, denied that it was a retreat. He said that Lehman's letter reflected exactly what he had said earlier, namely that "any EST that can be used as a probe is patentable subject matter".

Meredith Wadman

LHC and space station get funding strings

[WASHINGTON] A committee of the US House of Representatives gave backhanded endorsements to two controversial international programmes last week, authorizing US funding for both the International Space Station and the Large Hadron Collider (LHC) at the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland, but in each case attaching conditions to its approval.

The endorsements are not definitive, as a Senate committee must also have its say along with appropriations committees in both houses. But they do provide an indication of policy direction. In particular, the LHC vote represents defeat for a bid to cut funding completely, but still reflects congressional caution.

The committee voted unanimously to require the National Aeronautics and Space Administration (NASA) to meet strict requirements if Russian participation in the space station is to continue. In an amendment to a bill authorizing spending on civilian space science in 1998 and 1999, the committee barred NASA from transferring money to Russia or its contractors for work on the space station that the Russians have pledged to finance.

The amendment also requires NASA to certify each month whether or not the Russians are on schedule with their obligations to provide promised hardware, and to detail

contingency plans where they fail. It requires President Bill Clinton to tell Congress by the beginning of August whether Russia's promised hardware will be in place for the service module launch date of December 1998. And it forbids US astronauts from long-term stays on the Russian Mir spacecraft until NASA certifies that it meets US safety standards.

The amendment was jointly sponsored by James Sensenbrenner Jr (Republican, Wisconsin), the committee chairman, and George Brown (Democrat, California), the senior Democrat on the committee. Sensenbrenner made it clear that his continued support for the space station was granted reluctantly, arguing that NASA and the White House were in "denial" about Russia's ability to meet its obligations.

"There will be opportunities yet to remove the Russians from the programme if the station is not put onto a better track," he said, but added that such a decision would at present be "premature". Meanwhile, he called the amendment "an appropriate and measured step".

Funding for the LHC survived by eight votes an attempt to kill it by Joe Barton (Republican, Texas), who described the acronym as standing more appropriately for "Let's Have [US] Cash". Barton said he was opposed to "paying ransom for our scientists to participate" in the LHC, recall-

ing what he termed the European failure to support the ill-fated Superconducting Super Collider, which was to have been built in Texas.

The Department of Energy had asked for \$450 million — \$100 million of it in 1998 and 1999 — to help Europe build the LHC, which is due for completion in 2005. Instead, the committee stipulated that none of the funding for US high-energy physics should be used for the LHC unless the Secretary of Energy had reported to the committee and its Senate counterpart on the impact of US support for the LHC "on the operations and viability of United States high-energy and nuclear physics facilities". Sensenbrenner is to visit CERN this week.

In a separate vote, the committee voted by 18 to 8 to eliminate a \$10-million increase in the \$131-million budget for the Stanford Linear Accelerator Center. Vern Ehlers (Republican, Michigan), a physicist by training, sponsored the amendment cutting the money, arguing that such earmarking for specific facilities were inappropriate.

Overall, the committee approved bills that increased 1998 funding for the National Science Foundation by 7.2 per cent over 1997 — slightly more than the 7 per cent that science lobbyists had been seeking (see *Nature* 385, 103; 1997) — to \$3.5 billion.

Meredith Wadman

Creationist 'Ark' trial closes early after judge narrows focus

[SYDNEY] Australia's creationist trial ended earlier than anticipated last week after its scope, expected to encompass science education and the relationship between science and religion, had been considerably narrowed by Judge Ronald Sackville.

Sackville ruled as irrelevant much of the affidavits of two potential witnesses, Eugenie Scott of the US National Center for Science Education, and Edwin Byford, an Anglican theologian from Broken Hill, New South Wales. Neither was called to give verbal evidence on behalf of Ian Plimer, a geologist at the University of Melbourne.

Plimer has been challenging claims made in 1992 by Allen Roberts, an 'archaeological consultant' from Sydney, to have found "scientific evidence" for the remains of Noah's Ark at Akyayla in eastern Turkey (see *Nature* 386, 638; 1997).

Cross-examined by Plimer's barrister, Stephen Walmsley, Roberts distanced himself from Ark Search, the association that has promoted Roberts's lectures and sold tapes and brochures bearing his copyright. Linking Roberts with trade or commerce has been crucial to Plimer's allegations that Roberts and Ark Search engaged in

misleading and deceptive conduct.

Roberts holds a doctorate in Christian education from a peripatetic Bible college in Florida. Plimer's team argued that Roberts lacked the qualifications to conduct scientific tests for evidence of 'a great boat' and its human and animal passengers, and indeed had not carried out any tests.

Roberts admitted, in a written response to questions, that his claims to this effect were incorrect. He explained that when he had said during his lectures that "we found" fossilized remains he was referring not to himself but to a "fraternity" of Ark searchers. This group included two Americans, his supporter, Ron Wyatt of Nashville, Tennessee, and his opponent David Fasold.

Fasold, a former marine salvager from Oregon, is alleging that Roberts had breached his copyright in publishing a drawing of the 'Ark'. But this was denied by the artist Bruce Midgley, who had drawn a similar diagram for Roberts' brochure. Fasold is seeking financial damages.

Plimer is hoping that the judge will grant an injunction restraining Roberts from any further commercialization of his claims. Plimer believes such an order would act as a



deterrent to the efforts of fundamentalists to have 'creation science' taught alongside evolution in schools.

But Sackville expressed doubts about the use of the courts to adjudicate between competing ideas. "We have schools in this country that teach creation science or whatever," he said. "Are we going to protect all children from hearing these ideas, however ill-conceived Professor Plimer and others may think they are?" This verdict is expected in a few weeks.

Peter Pockley

Labour keeps its supporters guessing

[LONDON] Britain's Labour party last week revealed details of a National Endowment Fund for Science, Technology and the Arts (NESTA), which it promises to create if it wins next week's general election. It would initially be funded with capital from the National Lottery.

The new fund is one of the few firm commitments to have emerged from the party, which is widely expected to form Britain's next government, in a campaign marked more by rhetorical hand-waving about the importance of science being "at the centre of government" than by firm proposals about how this will be put into practice.

Even details of how NESTA — described as "the bank for British genius" — will operate remain sketchy. Labour officials say they hope that part of the fund's income will come from patent rights, either donated by individuals or corporations, or from research it has sponsored.

The model they use is the way in which the tail-end of the rights to the children's book Peter Pan were left by the author J. M. Barry in his will to Great Ormond Street Hospital for Sick Children. But they also admit that "we will have to see how things work out".

Such uncertainty is reflected more broadly in Labour's policy for science. A statement last week, for example, spoke of the need to encourage UK scientists working abroad to return to Britain. Indeed, Tony Blair, the Labour leader, announced that several had promised to do so if Labour wins power.

But no such individuals have been named, and several of those quoted in the party's press releases merely said that, while supporting the overall thrust of the party's policies, they are waiting to see how these are put into practice.

Labour's election strategy has caused frus-

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tration in the scientific community. Politically, however, it seems to have done the party little damage; an opinion poll by the *Times Higher Education Supplement* found that 64 per cent of university academics intended to vote for Labour next week, an increase from 57 per cent in the last election in 1992.

In contrast, 10 per cent plan to vote for the Conservatives, who form the present government under John Major, a drop from 17 per cent last time. Almost twice that number plan to vote for the smaller Liberal Democrat party, whose best hope is that it might — in the unlikely event of a close contest — hold the balance of power.

One reason for Labour's popularity in the academic community appears to be a widespread belief that a change of government will open up new opportunities, even if they have yet to be detailed. The reaction to the NESTA proposal already seems to have tapped some of this optimism.

Party officials stress that any money provided for science through the fund is not intended as a substitute for more conventional sources of support, in particular that which is distributed through the higher-education

funding councils and the research councils.

Nevertheless, the proposal was welcomed by the Committee of Vice-Chancellors and Principals because it would allow lottery funds to be used to address the problem of researchers "forced to use old equipment and work in poorly equipped laboratories".

Others say that their enthusiasm for a change in government lies in a conviction that a healthy science base requires a level of state intervention that Conservatives find difficult to accept. "I feel that a Labour government would bring a greater appreciation of the value of public institutions and of public sector activities," says Sir Martin Rees, professor of astronomy at the University of Cambridge. "There would be less of a tendency to feel that everything has to be run as a business."

The present government — in science, as more broadly — is fighting the election on its recent record, and is not totally without supporters in the scientific community. But such voices appear to be in the minority. Indeed, most observers feel that the main question now is not whether Labour will win next week's election, but what it will do once it does. And the lack of such details in the party's manifesto, has only fanned speculation.

The party has said it will not create a ministry of science. But one move that some feel it may take is to appoint key scientific figures to 'life peerages' to act as spokesmen in the House of Lords, as it is entitled to do in the traditional dissolution honours list.

Rees himself, who has been actively promoting greater public understanding of science, is seen by some as a possible candidate. Another figure widely expected to play a prominent role in a Labour administration is Sir Tom Blundell, until recently chairman of the Biotechnology and Biological Sciences Research Council.

Although Blundell — an active Labour member of the Oxford City Council in the early 1970s — has only lately become professor of biochemistry at the University of Cambridge, there are some who are keen that he should use his experience to play a central coordinating role in whatever policy the Labour party might put into place.

David Dickson

Return to Unesco beckons for Britain

[LONDON] Britain could find itself back in the United Nations Educational, Scientific and Cultural Organization (Unesco), which it left in the mid-1980s in protest at mismanagement and excessive administrative costs, if the Labour party wins next week's general election.

Labour party officials say that in principle they would like Britain to return "with the minimum of delay". This may not be immediately, as the decision will depend partly on a review of UK membership of all UN agencies.

Indeed, there is a note of

caution in the manifesto statement that it is Labour's "aim" to rejoin, after it has considered how this can be done "most effectively". It will also be necessary to decide where the membership subscription — which at current rates would be about £10 million (US\$16 million) a year — should come from.

The most likely source would be from reduced funding for bilateral aid programmes financed by the Overseas Development Administration, which Labour has promised to re-establish

as an independent agency

The United States remains outside Unesco, which it also left in 1984, with opposition to the agency firmly entrenched in conservative circles. However, a substantial body of Democrats, led by a former secretary of state, Cyrus Vance, favour rejoining.

Labour officials now say that "we want to be very much part of the reform process", pointing out that education is already at the top of the party's domestic political agenda.

D.D.

Computer sanctions 'could affect US weapons research'

[WASHINGTON] A US Department of Energy (DoE) official last week warned a congressional committee about the possible side-effects of imposing sanctions on a US supercomputer manufacturer for selling computers to a Russian nuclear weapons laboratory.

Victor Reis, assistant secretary for defence programmes, was speaking to the House of Representatives national security procurement subcommittee. He asked for restraint in the government's investigation of Silicon Graphics Inc. (SGI), in case it damages DoE's work with the company in developing nuclear weapons simulations.

The investigation is examining whether the shipment of four high-performance computers to Russia last year violated export control laws. But Reis suggested to the committee that, "should sanctions or punishments for proven violations be considered, such measures be carefully structured so as not to undermine the US [nuclear weapons] stockpile stewardship programme".

SGI, which owns the supercomputer pioneer Cray Research, admitted in February that it had shipped several high-performance computers to Russia without export licences. The company's chairman, Edward McCracken, said its officials believed licences were not required, as the computers were thought to be for civilian use. But he acknowledged that they should have been more diligent in finding out their use.

Victor Mikhailov, the Russian Minister of Atomic Energy, said in January that the computers would help to maintain the reliability of Russia's nuclear arsenal. He claimed they would boost by 10 times the computational capability of Chelyabinsk-70, the once-secret nuclear design laboratory.

SGI is the partner of Los Alamos National Laboratory in the Accelerated Strategic Computing Initiative, an ambitious effort by DoE to increase dramatically over the next decade the computational capabilities of its weapons laboratories. The effort is considered the backbone of DoE's "science-based stockpile stewardship program", which is aimed at ensuring that ageing nuclear weapons will work properly.

Last October, DoE and Los Alamos announced a \$110-million deal for Cray Research to develop and build what they said will be the world's most powerful computer, with a peak performance of three teraflops (a thousand billion floating point calculations per second). When combined with a separate system planned for the laboratory, the peak performance will be rated at four teraflops.

David Kramer

Human origins centre pulls up roots in move to Arizona

[BERKELEY, CALIFORNIA] The Institute of Human Origins is to leave Berkeley and affiliate with Arizona State University this summer, according to the institute's founder Donald Johanson, discoverer of the 3.2-million-year-old 'Lucy' fossil skeleton in Ethiopia.

Johanson set up the institute in 1981 as a non-profit, multidisciplinary research organization dedicated to "the recovery and analysis of the fossil evidence for human evolution and the establishment of a chronological framework for human evolutionary events". He will take a team of five scientists and the institute's programme coordinator to Arizona, where they will develop a multidisciplinary palaeoanthropology programme within the anthropology department.

The institute's departure will be a loss to the San Francisco Bay Area research community, where it acted as a magnet for visits from internationally recognized scientists and was responsible for strong community outreach programmes. But personal conflicts over the past few years have taken their toll on ties with the University of California at Berkeley, home to Tim White, a palaeontologist and once a close friend and colleague of Johanson, and others.

Three years ago, philanthropists Gordon and Ann Getty withdrew their support because of an apparent disagreement with Johanson. The institute dismissed Paul R. Renne, a geologist, and seven other scientists, who then sued the institute and formed the Berkeley Geochronology Center.

At Arizona, institute scientists will teach courses as well as continue their research, accompanying students to fieldwork sites in



Africa for on-site experience. The institute hopes eventually to expand its activities and to add more researchers in early hominids. "This new alliance opens the doors of East Africa to our students and faculty, with new opportunities to participate in potentially historical research missions," says Gary Krahenbuhl, dean of the college.

Johanson says that the new arrangement will allow money raised by the board to go directly into research and graduate education, rather than covering overhead expenses. Arizona State has committed \$320,000 to cover relocation, renovation and an operating budget during the first year, with about \$285,000 earmarked for the institute annually after that.

Johanson says that he is looking forward to working with both graduates and undergraduates, and to bringing to Arizona graduate students from field-research host countries such as Ethiopia and Eritrea. "It's time to give some serious thought to who's going to pick up the search where we leave off," he says.

William Kimbel, the institute's director of science, and Kaye Reed, a palaeontologist, are to join Johanson in the department of anthropology. Robert Walter, a geochronologist, will develop an argon dating facility in the geology department, and Eric Meikle, a palaeoanthropologist, will remain responsible for the institute's public outreach and education effort.

Sally Lehrman

India takes French route to genome project

[NEW DELHI] India will collaborate with France on human genome sequencing under an agreement signed earlier this month between the Centre National de la Recherche Scientifique (CNRS) and its Indian counterpart, the Council of Scientific and Industrial Research (CSIR).

The agreement followed discussions between CSIR scientists and a French delegation led by Guy Aubert, director general of CNRS. It runs initially for three years, but is renewable by mutual consent.

Both sides have identified "genome sequencing and analysis" as a priority area for collaboration. "We expect that a project on sequencing the human genome will be launched this year," said a spokesman for the French embassy's science office.

CNRS has agreed to open up its genome sequencing facilities to Indian scientists. In turn, CSIR has offered its DNA fingerprinting facilities in Hyderabad and Delhi to French scientists. The list of shared facilities in the two countries will be updated as required.

Indian officials say the agreement became possible because of a clear understanding between both sides on the need to protect intellectual property rights. Under the agreement, results arising from the collaboration will be common property and the benefits will be shared equally.

In addition to genome sequencing, the two countries will cooperate on, drug development, oceanography and chemical sciences.

K. S. Jayaraman

Germany divided on a single academy

Quirin Schiermeier

Germany is developing plans for a national academy of sciences, but the country's regional academies and a learned society have differing ideas.

The German government has been asked to consider setting up a new national academy of sciences along the lines of the US National Research Council. But the suggestion is likely to anger Germany's many regional science academies which are competing to be chosen as reunified Germany's first national scientific voice.

The recommendation comes from Dieter Simon, president of the Berlin-Brandenburg Academy of Sciences. In a paper requested by Jürgen Rüttgers, minister for education and research, Simon says that setting up a new institution would put an end to the emerging public battle between the regional — or *Länder* — academies.

A national academy was first suggested by Chancellor Helmut Kohl during a speech to parliament in 1994. The idea was revived late last year and in February Simon laid out the options in his paper to Rüttgers.

Simon suggests that the new body be called the National Academic Convention. It could function, he says, as an *ad hoc* group of 30 or 50 scientists appointed by the German president, and be called upon to address problems proposed by the government, parliament or a scientific organization. The present period, before the start of the 1998 election campaign, could be the right time to push ahead with such a plan, Simon says.

But Germany's other *Länder* academies see things differently. They believe that their umbrella organization, the Conference of Academies, is exactly the sort of forum that is needed. They place their hopes on an internal paper of the research ministry which, they claim, takes seriously the suggestion of a national academy based on the organization.

Long history, strong rival?

The regional academies, however, have another rival in the shape of the Leopoldina Academy of Scientists, Germany's oldest learned society, formed in 1652 and based in Halle in east Germany. The Leopoldina considers its exceptionally long history a justification for forming the kernel of a national academy.

At its biennial conference in Halle last month, the Leopoldina's president, Benno Parthier, said that his academy "must not be passed over if a German national academy of sciences is to be established".

Simon says he agrees in principle,

acknowledging the tradition and reputation of the Leopoldina. But he doubts that it could adequately represent the whole spectrum of German science, because it is exclusively focused on medical and natural sciences.

According to Simon, designating the national voice to an existing regional academy could avoid a constitutional conflict over the traditional *Länder* autonomy in cultural affairs. But he sees only the Berlin-Brandenburg Academy, formed in 1993, as suitable for the purpose.

"Being an efficient working academy with a nationwide and international membership, Berlin-Brandenburg is much more than just a learned society of elderly men," says Simon. "And, unlike other *Länder* academies, we are professionally organized, with a full-time president."

Decentralized and reunified

Germany has never had a true national science academy, and after the Second World War the prospect of one being formed became unlikely. West Germany's new decentralizing constitution placed responsibility for all cultural matters firmly in the hands of the newly created regional — or *Länder* — governments, six of which either inherited or created their own academies.

As a result Germany has no single voice that can represent national opinion on scientific issues either to the outside world or to German politicians. This is a point of concern for both Rüttgers and many scientists.

Reunification left Berlin, the former and future capital, with two academies of science. A relatively new, and very small, west Berlin academy fell victim to the political animosity of reunified Berlin's socialist-Green party coalition, which closed it down in 1991. The coalition also closed down the learned society section of the former East German Academy of Sciences, despite a clause in the reunification treaty that specified that it should be preserved "in appropriate form".

The Senate of Berlin considered that the "moral damage" imposed by the Communist regime, which used the academy as a political tool, could not be repaired. Rubbing salt into the wound, it gave the learned society's valuable archives and libraries to the Berlin-Brandenburg academy when it was founded in 1993. This continues to be a source of bitterness for eastern academics.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

The Leopoldina (above) is one of several bodies claiming a new role as a national academy.

Herbert Wöltge, a former spokesman for the east German learned society, compares the affair to the effect of a neutron bomb "killing the people, sparing the material assets".

The Leopoldina, on the other hand, came out of the Communist era with fewer scars. A supranational learned society with 940 members divided among 17 scientific and 17 medical sections, the Leopoldina has roots in all the German-speaking countries, and was generally believed to have come through with its moral integrity intact.

"The [former East German] government had regarded Leopoldina as a prestigious tool to demonstrate its supposed open-mindedness to the rest of the world," says Parthier. Because of this, it had a strong measure of independence and, unlike the learned society section of the East German Academy of Sciences, it was free to choose its members according to scientific merit rather than on the basis of loyalty to Communist doctrines.

Surveillance by secret police

But unpleasant truths have recently come to light — the Leopoldina had been the object of extensive surveillance activity by the STASI, the East German secret police. Parthier has found more than 5,000 pages of STASI records in the 'Gauck' office, where all STASI files are now archived and available for access by persons believed to be the object of surveillance. The files detail an 11-year campaign targeting the Leopoldina, which was code-named 'Komet'.

"We knew that all our meetings were infiltrated by informers, but we were naive enough not to see that even private conversations in our rooms were constantly bugged by the STASI," says Parthier.

The files show that the STASI became particularly active during visits from prominent west German scientists such as the physicist Carl Friedrich von Weizsäcker and the biologist Heinz Staab, former president of the Max-Planck Society. "Such persons were invariably sounded out by STASI informers under the guise of science," says Parthier. □

Lack of progress leads India to review role in chemical arms treaty

[NEW DELHI] India is threatening to withdraw from the United Nations Chemical Weapons Convention if key players among the 162 signatories do not ratify it before the treaty takes effect on 29 April. Only 72 countries have so far ratified the treaty, which bans chemical weapons. New Delhi was among the first to ratify it last September, but officials have now said the government will "review" the decision because the treaty as it stands "is shorn of both its key principles — representativeness and disarmament character".

India's objection is that it allows countries such as the United States and Russia to retain their chemical arsenals while making it necessary for treaty members to open up their chemical industry for intrusive inspection. India also feels that its security-related concerns are not answered by the treaty without the membership of Pakistan and China, which both have chemical weapons capability.

Meanwhile, US President Bill Clinton has issued a last-ditch appeal to Republican senators not to obstruct a bill ratifying the convention, which will be put to the vote this week. Clinton needs to secure 67 out of 100

senate votes. Assuming support from every single Democrat senator, he still needs votes from at least 22 Republicans. Tom Daschle, leader of the 45-strong Democrat contingent in the Senate, puts the president's chances as no greater than 50:50. The treaty will take effect regardless of US support, as it has already obtained the required minimum number of ratifications.

FBI laboratory 'requires urgent overhaul'

[WASHINGTON] The vaunted crime laboratory of the US Federal Bureau of Investigation (FBI), which examines 600,000 pieces of criminal evidence a year for federal, state and local law enforcement agencies, is in need of an urgent overhaul, according to a report released last week by the Justice Department. After an 18-month investigation focused on three units within the laboratory's scientific analysis section, the department's inspector general found "significant instances" of substandard analytical work, deficient practices and flawed scientific testimony.

The tainted evidence may affect the outcome of several high-profile cases, including the 1993 bombing of the World Trade Center in New York and the 1995 bombing of a government building in Oklahoma City. Even before the final report, the FBI had begun to make reforms,

including removing the directors of the laboratory's explosives and chemistry units, and moving to have the laboratory accredited by an outside body, the American Society of Crime Laboratory Directors.

CF gene tests backed for pregnant women

[WASHINGTON] An expert panel convened by the US National Institutes of Health last week recommended that pregnant women and those considering having babies should be offered genetic testing for mutations that cause cystic fibrosis, and that health insurers should cover the costs of testing.

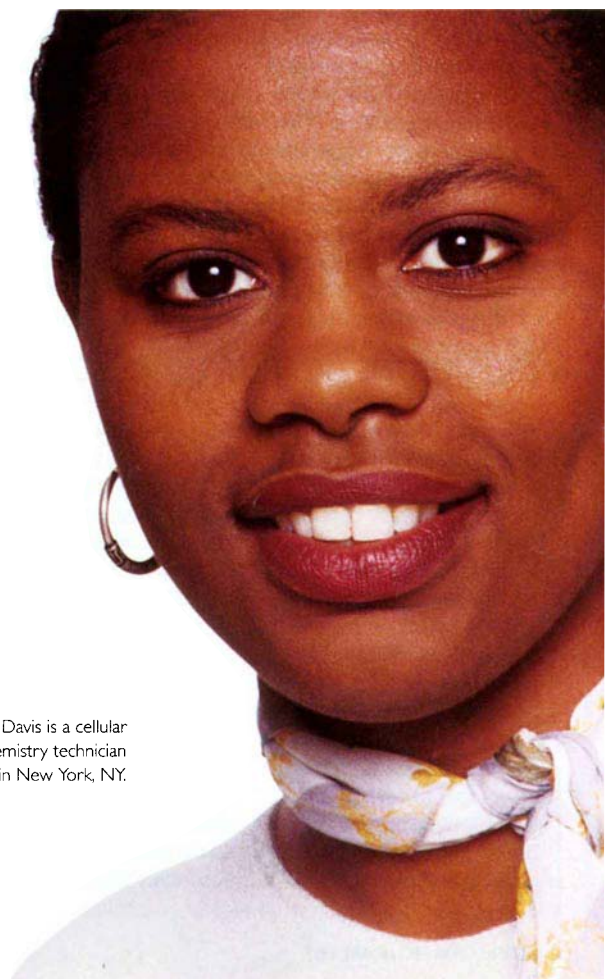
If implemented, the recommendations — aimed also at those with family histories of the disease — would mark the first broad-based population screening for carriers of genetic disease in the United States. The panel's recommendations are expected to carry considerable weight and could, for example, provide ammunition that could be used by patients to challenge insurers who refuse to pay for testing.

German funding agency to be led by biochemist

[MUNICH] Ernst-Ludwig Winnaker, head of the Centre for Genetics at the Ludwig Maximilian University in Munich, is to be

Obtaining **reliable**
PCR results means
using **Ready-To-Go**
PCR Beads

Terri Davis is a cellular biochemistry technician working in New York, NY.



No lack of individuality

Sir — Audrey Wells writes that “Confucian-influenced cultures such as China, Korea, Japan and Vietnam were not deeply permeated by Christianity and its concomitant emphasis on the importance of the individual” (*Nature* 386, 14; 1997). We contend that if lack of individuality is indeed the case, original Confucianism had nothing to do with it. The original *Rú Jiā* philosophers placed emphasis on individuality and self-examination. In *Lùn Yǔ* (Analects), we have: “the Master said, ‘When the multitude hate a man, it is necessary to examine the case. When the multitude like a man, it is necessary to examine the case.’” (James Legge’s translation for this and other quotations.) Mencius continued in this tradition, and *Měng Zǐ* has this passage: “I heard an account of great valour from the Master. It speaks thus, ‘If, on self-examination, I find that I am not upright, shall I not be in fear even of a poor man in his loose garment of haircloth? If, on self-examination, I find that I am upright, I will go forward against thousands and tens of thousands.’” This is a philosophy that encourages the individual to think and act independently.

Authoritarianism is also a trait unknown in the original writings of the Confucian philosophers. What they respected was not power or authority, but virtue. *Měng Zǐ* has the following: “Respect shown by inferiors to superiors is called giving to the noble the observance due to rank. Respect shown by superiors to inferiors is called giving honour to talents and virtue. The rightness in each case is the same.” And deference towards seniors is only half the story; there is also deference towards the young. In *Lùn Yǔ*, Confucius was recorded as having said, “A youth is to be regarded with respect. How do we know that his future will not be equal to our present?”

Before Galileo, Chinese science was much more advanced than science in Christendom — or anywhere else for that matter. The most authoritative scholar on the relationship between Chinese science and Taoism is probably the late Joseph

Needham. In his *Science and Civilization in China* (Cambridge University Press), he wrote that Taoism was the main driving force behind Chinese science, because of the Taoist view on the harmony between Man and Nature. He never associated Taoism with paradoxes, and it would be interesting to know how they are related to Taoism, because, to our knowledge, paradoxes have never been connected to Taoism.

Nevertheless, Needham also mentioned that Confucianism contributed to scientific developments as well, albeit to a lesser extent, by providing the necessary social infrastructure. In his magnum opus, one can examine these developments. It is clear that the ancient Chinese, at least up to the time of the Scientific Revolution, did not lack individuality or independence of spirit, two qualities (among many others) conducive to scientific development. We would further suggest that the Confucian ideals of self-examination and moral courage probably contributed to this individuality and independence of spirit. Needham also explored the possible reasons against a scientific revolution in China, and it appears to be less simple than just the lack of Christian-influenced individualism.

Lastly, Audrey Wells contends that “Confucianism does encourage self-discipline”. May we ask what philosophy or religion does not encourage self-discipline? Do Buddha, Socrates and Plato, or the Bible, the Koran and the Vedas not do the same? And is it statistically significant to generalize from Western musical education in Korea and Japan in the past 30 years or so to arrive at a general conclusion about a complex 2,500-year-old philosophy that has deeply influenced the whole of East Asia?

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Sequence is all there

Sir — In a recent News story about the sequencing of the *Escherichia coli* genome (*Nature* 385, 472; 1997) you said that “the unannotated Japanese sequence is a hybrid of more than one strain”; you also said “So far, however, only raw sequence data are available in the public database” (my italics in both cases).

In fact, since 24 January 1997, the

annotated sequence of almost all the *E. coli* genome determined by us has been in the public database. Our data, like those of Blattner *et al.*, are of the highest quality, and our analysis of sequence features has been even further extended.

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Contract researchers still out in the cold

Sir — A recent leading article (*Nature* 386, 201; 1997) stated: “The Natural Environment Research Council [NERC]’s enlightened policy is that any employee of more than five years’ standing should be permanent. Unfortunately, many such employees are still impermanent.”

While it is correct that many NERC employees with more than five years’ continuous service are still on fixed-term contracts (and are thus impermanent), your statement does not correctly summarize NERC’s policy. This policy is that (in theory) staff on fixed-term appointments approaching five years’ service will be reviewed, and, at the end of the contract, either will be offered an open-ended post or their position will be terminated. However, as the governing council of NERC insisted that the policy be implemented at a local level within NERC’s institutes, the policy is being interpreted very differently in different parts of NERC. At the British Antarctic Survey for example, the stated policy is that only researchers who are “high flyers” of “outstanding/ excellent ability” will be retained. This description is usually reserved for staff given a “box 1” mark on their annual personal review — approximately 5–10% of the staff — which doesn’t make the policy particularly attractive for the remaining 90% on fixed-term contracts.

The NERC policy may, in principle, seem enlightened, but it is the practice of this policy that matters. Directors within NERC are still more concerned to “maintain flexibility” by increasing the numbers of staff on fixed-term appointments than in creating an environment and career structure within which scientists can flourish over the longer term. As your leading article concludes: “Every means should be found to keep up the pressure on all employers who keep contract researchers... in a state of unjust personal disadvantage.” The trade unions within NERC, led by the Institution of Professionals, Managers and Specialists (IPMS) representing the scientists, continue to campaign for better terms and condition for those on fixed-term contracts, and it looks as if we will be campaigning for some time to come.

Tina Yates

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Value-added Frontiers programme

Sir — As a new member of the Council of Scientists of the Human Frontiers Science Program (HFSP), I was interested to read your recent leading article and News story (*Nature* **386**, 95 & 100; 1997).

As you correctly point out, some research requires major investment in one facility, and this must be funded internationally. CERN is the prime example, and it is good that Tokyo has agreed to contribute. However, although I agree that particle physics is qualitatively different from research on the brain, it is true that international collaboration can provide added value to national programmes. This is particularly true in a multidisciplinary research area such as brain research, where different countries may contribute complementary expertise. The HFSP, with relatively modest funds, achieves this superbly.

In the programme, the necessity for international collaboration is a major factor taken into account by review committees in assessing grant applications. Further, to demonstrate that the programme actually succeeds in its internationalist aims, we can cite the General Review, in which grant recipients and those just below the cut-off point were interviewed:

"Only three per cent of the unsuccessful applicants reported that they were able to carry out exactly the same research and collaboration without the HFSP grant. . . . Although roughly half the unsuccessful applicants said they were able to do some of the intended research and collaboration without HFSP support, they were able to do significantly less collaboration (or none) and the scope of the research was diminished. The remaining half reported that they were not able to carry out any of the proposed research or collaboration at all."

That there is an increasing globalization of scientific research seems without doubt. The foregoing testimonies indicate that the HFSP is having an important impact on international science over and above the possibilities offered by the national funding agencies.

In Richard Nathan's News article, an anonymous British researcher complains that it is easier to obtain funds in the United Kingdom than from the HFSP and that funds should not therefore be diverted. This is a Catch-22 argument. The review committees consider that many of the applications not funded do fulfil the requirements of scientific quality and the need for international collaboration. The ability of the HFSP to carry out a 'value

added' role, in a way that national agencies do not, is a powerful argument for an increase in financial support.

Tom Blundell

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Ginkgo thrives

Sir — A recent News story described a report which concludes that the increasing popularity of herbal medicine in the Western world is threatening its natural base, medicinal plants growing in the wild (*Nature* **385**, 570; 1997). It also refers to Germany as being the largest consumer of these medicinal plants, and focuses mainly on the ginkgo tree as an example of such a plant threatened by overuse.

This company, Dr Willmar Schwabe Arzneimittel, is a leading manufacturer of plant-based pharmaceuticals. Ginkgo leaves are indeed the source of the company's most outstanding product. It is a highly refined extract 'Egb 761', the active ingredient of a preparation sold the world over under trademarks such as Tebonin, Tanakan, Tebokan, Tebofortran and Rökan. These products are successfully used in the treatment of cognition and memory impairment as well as vascular and Alzheimer-type dementia.

To meet the rising demand for ginkgo leaves, we established our own ginkgo plantations near the Atlantic coast of France and in South Carolina in the United States in 1982, together with the Beaufour-Ipsen Group, our French partner. Twenty-five million trees growing on 1,000 hectares of land on these two sites are producing close to 4,000 tonnes of dried leaves a year. (The author mentions a figure of 2,000 tonnes being consumed in Germany.) In addition, more than 25 million trees are cultivated in China for the same purpose. Specific cultivation techniques for propagation, nursing and harvesting have been developed to meet the specific needs of this unconventional crop. In this way, we do our best to safeguard the natural resources of ginkgo. At the same time, we are assured of a raw material of high standard, produced under ecologically sound and well monitored conditions.

Ginkgo is also cultivated in Japan and South Korea, and is used worldwide as an ornamental tree which, incidentally, is resistant to urban pollution. For some time it was disputed whether *ginkgo biloba* still occurred under natural conditions but Chinese researchers have shown that it grows in natural mountainous forests in

the Chinese provinces of Anhui, Zhejiang and Guizhou. So the ginkgo tree is by no means a threatened species. If it has become less abundant, there are probably other reasons than the mere gathering of its leaves.

To develop a new product in the pharmaceutical industry costs millions of dollars before it even reaches the market. It would not make sense for us to make such large investments if we were not sure of the resource base. Any investment based on a resource plant threatened by extinction would be meaningless.

Wilhelm Schmid

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Todd's achievement

Sir — Lord Todd ("Tod Almighty" to his students) would have appreciated John Maddox's obituary of him (*Nature* **385**, 492; 1997).

Allow me to make two corrections. Todd did not isolate the active constituent in cannabis. He isolated cannabinal, which is barely active. Most of the cannabinal in hashish (or possibly all of it) is an oxidation product of other constituents. Todd knew this and mentioned it in his autobiography.

The active constituent of cannabis is Δ -9-THC which Y. Gaoni and I isolated and elucidated the structure of in the 1960s (*J. Am. Chem. Soc.* **86**, 1646; 1964). We and others did quite a bit of work on it and today there are thousands of papers on THC. Burstein and I reported the first step of THC metabolism in *Nature* (**225**, 87–88; 1970) and *J. Am. Chem. Soc.* (**92**, 3468–3469; 1970).

When we identified arachidonoyl-ethanolamide (anandamide) as the endogenous brain ligand that binds to the cannabinoid receptor (*Science* **258**, 1946–1949; 1992) Todd was delighted.

Raphael Mechoulam

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correction

Christine Clayton's letter (*Nature* **386**, 432; 1997) about spelling mistakes and grammatical errors contained a grammatical error introduced in the *Nature* office. The fourth sentence read: "Last year, a paper I had submitted was returned by the journal concerned complaining that it was 'strewn with spelling mistakes and grammatical errors.'" It should have read: "Last year, a paper I had submitted to a North American journal was returned with accusations that it was 'strewn with spelling mistakes and grammatical errors.'" — Editor, *Nature*.

A man for our season

Science is in a parlous state — passing fashions, a star system and the new cult of management have combined to strangle originality. The ethics of V. B. Wigglesworth offer a cure.

**Peter A. Lawrence and
Michael Locke**

*And as our vineyards, fallows, meads and hedges,
Defective in their natures, grow to wildness,
Even so our houses and ourselves and children
Have lost, or do not want to learn for want of time,
The sciences that should become our country.*

Henry V, V, ii 54–58

Throughout the world, but especially in Western Europe, North America and Japan, the sciences are threatened by changes in practice and by erosion of principles. An appreciation of the philosophy that guided the research life of Vincent Wigglesworth (1899–1994), the great insect physiologist^{1,2}, may help to find a cure for this malaise.

Wigglesworth was a shy man, gentle but single-minded, with a sharp enquiring intelligence. His passionate childhood interest in insects led to science, a medical degree and a lifetime of research on all aspects of insect physiology and morphogenesis. He published 264 papers between 1923 and 1991, in all but 19 of which he was the only author. He wrote nine books and updated his superb text *The Principles of Insect Physiology* seven times. Wigglesworth was extraordinary, both for the number and range of his discoveries, and for the way he achieved them. He started his career when technical know-how and expensive equipment were less important than a clear mind. His approach to research can teach us much that is crucial to the practice of science.

Like Darwin, Wigglesworth believed that the primary purpose of a scientist is to find illuminating solutions to problems: "Except in so far as they contribute to theories and generalisations the scientific mind is not interested in facts"³. Many of his discoveries came, not from the narrow preoccupations of fashion, but from keen observation of experimental material, a much more unpredictable and therefore broader source. Nowadays the spotlight cast by fashion, and a star system that highlights the contributions of the few, combine to limit adventures in ideas. Modern science is becoming like the *mêlée* at a children's party where groups rush hither and thither after any kid who shouts loud enough.

Wigglesworth belonged to a formal and genteel age. He would not talk about his work in progress, nor would he show interest in the unpublished work of others; because he viewed much of what was printed as wrong or misleading, and rightly so, he thought there was little point in paying attention to unpublished material — it was even less likely to be

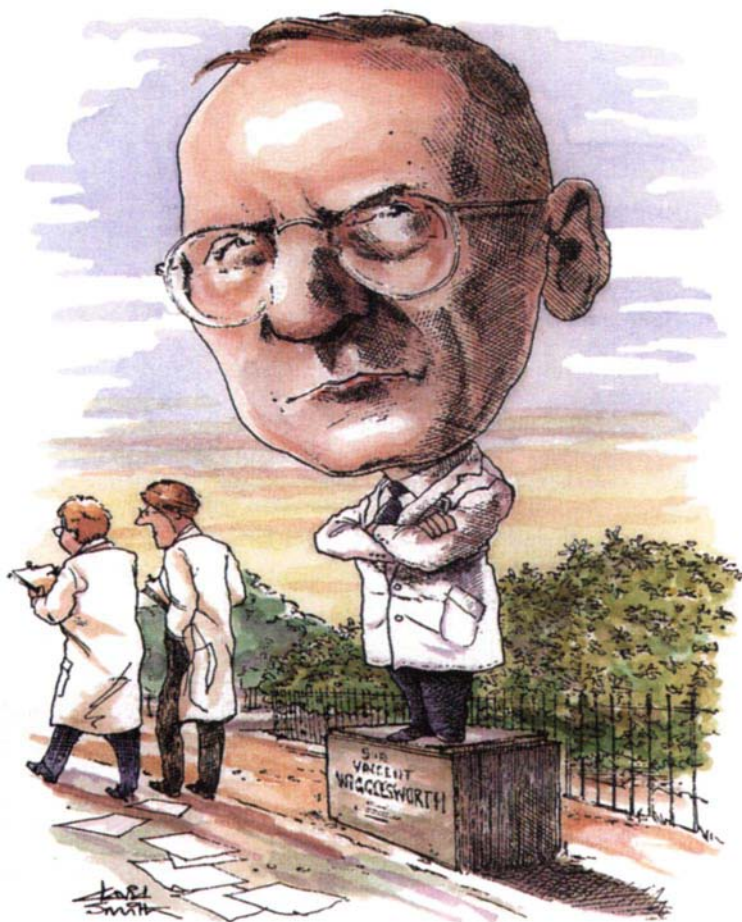
correct. By contrast, modern science thrives on rumour; the stars in each field travel the world, talk at conferences, and publish and republish in the secondary literature. The result is a homogenization of opinion that can stifle originality; a reduction of the tendency of independent schools of scientists, particularly in different countries, to evolve their own approaches to problems.

Journals can also limit originality. Apart from pandering to fashion, editors seem increasingly to be relinquishing what should be their decisions to reviewers⁴, and reviewers tend to dislike competitors' hypotheses. Short, clear papers with unifying ideas, such as that of Watson and Crick⁵, are out of favour, and detail is becoming confused with profundity. Scientists, who like artists should be trying to build pictures of ideas, are forced into secure but dry descriptions, with any implications hidden by mountains of data. To quote Wigglesworth again: the BBC had returned his script "...asking whether I could let them have something more profound. I replied that I could not promise to write a script that was profound,

but if that would be more acceptable, I could easily make it more obscure"⁶.

Wigglesworth was very correct: he rarely used a first name, even with colleagues he had worked with for decades. But when he wrote, it was as if he became unshackled from convention and used a simple direct style, free of jargon and acronyms. Nowadays we may be less regimented in dress and manners, but our writing has degenerated into an impersonal coded newspeak that leaves the authors' characters undetectable, their ideas disguised. As Montgomery puts it, "Any point at which there emerges something resembling a truly personal or literary style in a technical article is commonly considered to be a point of failure"⁷.

Wigglesworth worked alone — he taught by example and was unwilling to do experiments by proxy. He wrote papers from his own results and was never faced with the dilemma of principal investigators who sometimes have to decide between their own prejudices and the findings of a junior colleague. Not knowing exactly how information is acquired makes it easier to publish



careless or even fraudulent data. Nowadays, heads of laboratories include their own names on papers, claiming ownership of the work done by students or postdoctoral fellows. At its best, the addition of principal investigators' names gives authority, for they are presumed to have checked the results and to vouch for the conclusions. In practice, their function is increasingly managerial, manipulating multiauthorship and name order as part of the politics of science.

Author sequence may be permuted to allow each young scientist in a large group to be first author, so that the sequence no longer signals responsibility. Others with tenuous claims to authorship, and little or no knowledge of the work, may clamber on board (A did the biopsy, B donated an antibody, C checked the statistics, D's technician collected the samples). Papers tend to be written when people leave or need a job, or when a grant renewal becomes due, rather than when the results are ready for publication. The consequence is that many papers have become competitive tokens for insertion into grant-dispensing gambling machines rather than bricks in the edifice of science.

Wigglesworth worked with the introvert intensity characteristic of many creative people. We might now stigmatize him as a loner. Selection committees would judge him lacking in the forceful leadership, managerial skills and salesmanship they suppose to be required in our tough world of entrepreneurial science. There is a place for aggressive scientific publicists, teachers and laboratory directors, but history has shown that original and creative minds are rare, and should be looked for also among the reserved and introspective. We should recognize that the scientific establishment needs many kinds of talent and modify selection procedures appropriately.

Wigglesworth's attitude to students was motivated by his belief that a fresh mind was an asset that should not be squandered, as well as by his wish to be free to do his own research. He recognized that students need to learn how to be independent, so he gave them independence. He did not author their papers. They learned to write early, to take responsibility for the correctness of their results, and to defend their interpretations. Learning how to report results should not be separated from learning how to get results; both are integral parts of becoming a scientist. These days the principal investigator often supervises students and postdoctoral workers very closely. If we liken the search for knowledge to the search for gold, many are trained to be miners rather than prospectors. They leave their mentor and become 'independent', but continue on the same line with groups of their own. Some may go through their entire careers without really choosing their own field of research.

Group science carries the threat of inertia, but Wigglesworth was never troubled by

The rise in the dictatorship of the manageriate is lowering the status of scientists, demoralizing them and shredding their sense of purpose

such a problem. For example, although he grew up in a period when geneticists and embryologists rarely talked to one another, and at first had little interest in genetics, he came to understand how relevant it is to his favourite subjects, pattern formation and morphogenesis. He became an ardent advocate for the genetic approach to development⁸. No one can progress far in science without changing ideas and research direction. Yet, in large groups, commitments to grant objectives can make big jumps and opportunistic research too risky. Moreover, careers depend on publications which come more securely from overlapping doctoral theses than from innovation.

Changes in funding practices are at the root of much of the malaise afflicting science. Take Britain — in Wigglesworth's day, the research councils recognized the excellence of the individual's record as a sufficient basis for scientific support; his productivity, and the diversity and originality of his papers, show the effectiveness of the research council system under which he worked. Things are different now. Grants are awarded on the basis of frequent short-term assessments, a method that discourages adventure. Ed Lewis, for example, would have had difficulty getting support for his Nobel prize-winning work in developmental genetics had he been subject to present-day criteria⁹ — his key papers were produced about once a decade.

Wigglesworth had a healthy view of science administration: he thought administrators should see their prime purpose to be the facilitation and encouragement of research and that "Anyone who has tried both, knows that administration is so immeasurably easier than research that it becomes the line of least resistance and that is why research needs encouragement"¹⁰. Now, numbers of papers, 'quantitation' and audit are emphasized, bedevilling creative activity in the arts as well as in science. The root cause lies in the new cult of management which puts administrators at the top of a hierarchy, confusing management with leadership.

As a result of this trend, an organization with a mission in medical research had its scientific posts evaluated by consultants who devised a questionnaire that rated administrative skills as the most important; and salary disclosures at a group of universities showed that some minor deans earned more than many distinguished scientists. The

solution to this problem lies in resisting the tendency of bureaucrats to increase their status and income and in making sure that administration does not compromise the scientific mission. This may not be easy, but democracies have solved the problem in an analogous situation. Police need authority: a police officer may arrest a prime minister, but this does not result in the pay of a police officer being greater than that of those over whom he has authority. Remuneration for administrators and research coordinators needs to be viewed in the same way; it should be of a lower order than that of productive researchers, who by their discoveries can bring major benefits to society.

In both Western Europe and North America, good students are being diverted from science to careers that are more lucrative. Other countries, notably in Eastern Europe and Asia, where higher education is still a way to the top and which recognize the social value of scholarship, now supply the graduate students. The rise in the dictatorship of the manageriate is lowering the status of scientists and scholars, demoralizing them and shredding their sense of purpose.

Clocks cannot be turned back. But pendulums can swing, and we hope that they will start to swing soon in the matters we raise here. Could grant reviewers attach more value to independence in the training of the next generation, favouring projects where students are likely to publish on their own? Could editors invoke Darwin's dictum that "all observation must be for or against some view if it is to be of any service"¹¹, and insist that publication in science involves the art of telling a story? Can politicians be educated to know what science is and to value it for its long-term benefits, rather than putting their trust in the new cult of short-term management? We should all reflect on the conditions that allowed some people, Wigglesworth among them, to make great scientific contributions. □

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1. Locke, M. *J. Insect Physiol.* **40**, 823–826 (1994).
2. Locke, M. *Biogr. Mem. R. Soc.* **42**, 541–553 (1996).
3. Wigglesworth, V. B. *Parasitology* **47**, 1–15 (1957).
4. Henneberg, M. *Nature* **384**, 401 (1996).
5. Watson, J. & Crick, F. H. C. *Nature* **171**, 964–967 (1953).
6. Wigglesworth, V. B. *Ann. Appl. Biol.* **60**, 1–10 (1967).
7. Montgomery, S. L. *The Scientific Voice* (Guilford, New York, 1995).
8. Wigglesworth, V. B. in *Insect Polymorphism* (ed. Kennedy, J. S.) 104–111 (Symp. R. Entomol. Soc. Lond., 1961).
9. Lawrence, P. A. *The Making of a Fly* 211–215 (Blackwell Scientific, Oxford, 1992).
10. Wigglesworth, V. B. *The Advancement of Science* **26**, 1–8 (1950).
11. Darwin, C. in *More Letters of Charles Darwin* (eds Darwin, F. & Seward, A. C.) Vol. 1, 195 (Appleton, New York, 1903).

The AM and FM of calcium signalling

Michael J. Berridge

Cells respond to external signals by transducing the information into internal messengers such as Ca^{2+} . The information-processing capability of the Ca^{2+} signalling system is enhanced by the well-known engineering principles of amplitude and frequency modulation.

The question I am asked most frequently about Ca^{2+} is how such a simple messenger can regulate so many diverse cellular processes, such as fertilization, secretion, contraction, proliferation, neural signalling and learning. With much hand waving, I usually speculate that such diversity might be achieved by operating through different amplitude, spatial or temporal modes. This is not too fanciful, because we had established some time ago that insect salivary glands use frequency modulation (FM) to control fluid secretion¹.

Now Dolmetsch *et al.* (page 855 of this issue²) have clearly demonstrated that differential gene transcription in B lymphocytes, the immune system's antibody-producing cells, is achieved through amplitude modulation (AM) of this ubiquitous Ca^{2+} signalling system (Fig. 1). The basic observation is that low concentrations of Ca^{2+} activate the nuclear factor of activated T cells (NFAT) and the so-called ERK pathway, whereas a much larger elevation stimulates a different set of transcriptional regulators (for example NF- κ B and c-Jun kinase). This differential gene activation using an AM signalling mode provides an elegant explanation of how B cells, which can be separated into two groups, respond differently when they are presented with the same antigen³. Naive cells, which have had no previous contact with the antigen, produce a large Ca^{2+} signal which induces proliferation and positive selection. On the other hand, self-tolerant B cells that have responded previously and are thus tolerant to this particular antigen, generate a much smaller signal which fails to stimulate proliferation; instead, the cells display negative selection by blocking plasma-cell differentiation and antibody secretion (Fig. 1). The same B-cell receptor (BCR) uses the same second messenger, Ca^{2+} , to control two separate cellular processes by using an AM form of signalling.

It seems, therefore, that Ca^{2+} can increase its signalling potential by operating through either AM or FM modes. With regard to the FM mode, the Ca^{2+} signal often appears as regular oscillations whose frequency changes with the concentration of the incoming signal. This FM signalling mode is used to control the rate of specific cellular

processes such as fluid secretion by salivary glands¹, glycogen metabolism by liver cells⁴ or neuronal differentiation⁵. In the last example, expression of the neurotransmitter GABA and K^+ channels is tuned to the Ca^{2+} spike frequency found in the cell body

whereas neurite extension is controlled by a different frequency.

As yet, there is no indication that cells employ FM to stimulate different calcium-dependent processes within the same region of the cell. This is why the discovery that lymphocytes use AM to activate nuclear signals independently of each other is such an important development in the field of Ca^{2+} signalling. A necessary prerequisite of AM signalling is that the various Ca^{2+} -dependent events must have different affinities for Ca^{2+} , and this needs to be shown for the various transcriptional events depicted in Fig. 1.

To appreciate how cells use AM and FM modes of signalling, it is necessary to explore the properties of the Ca^{2+} messenger system in a little more detail. When cells respond to hormones, growth factors or, in the case of lymphocytes in Fig. 1, the arrival of an

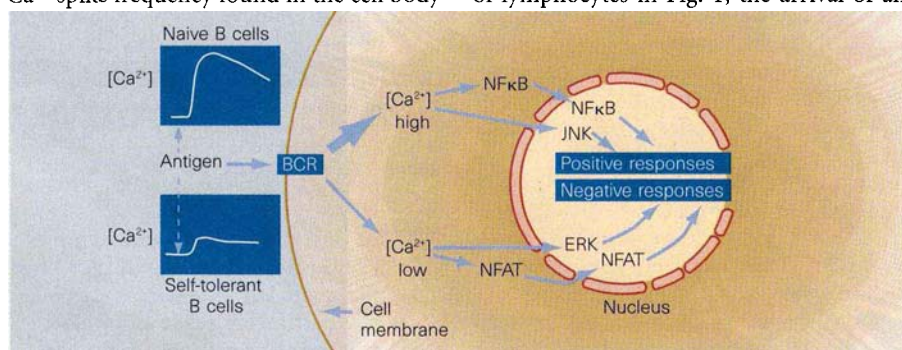


Figure 1 Differential gene activation in B cells through amplitude modulation of the Ca^{2+} signalling pathway responsible for transmitting information to the nucleus. As Dolmetsch and colleagues² show, depending on the cell (naive or self-tolerant), antigen acting on the B-cell receptor (BCR) can stimulate either high-intensity or low-intensity Ca^{2+} signals (see insets).

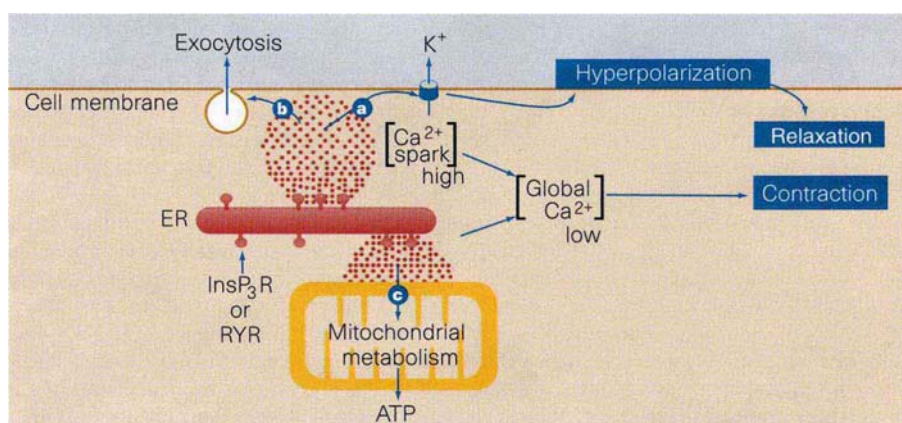


Figure 2 Local Ca^{2+} signalling through elementary events such as 'sparks' and 'puffs'. Groups of either inositol-1,4,5-trisphosphate (InsP_3) receptors or ryanodine receptors (RYRs) located on the endoplasmic reticulum (ER) release localized bursts of Ca^{2+} , giving rise to elementary events such as sparks where the local concentration is very high before the Ca^{2+} diffuses away to contribute to the global Ca^{2+} signal. a, An elementary event located near the plasma membrane in smooth muscle cells opens high-affinity, Ca^{2+} -sensitive K^+ channels to give small, spontaneous, outward currents which can sum to cause the muscle to relax by hyperpolarizing the membrane⁹. It appears, therefore, that smooth muscle uses AM signalling, in that low-intensity global signals stimulate contraction whereas the high but localized concentrations within the elementary events near the membrane cause relaxation. Depending on how it is presented, Ca^{2+} can stimulate either contraction or relaxation. b, Release of granules from pituitary cells appears to be triggered by InsP_3 -induced release of stored Ca^{2+} (ref. 10); release might be triggered by the high concentrations associated with the elementary events. c, Energy metabolism by mitochondria can be activated by the high concentrations of Ca^{2+} associated with the elementary events that give rise to typical Ca^{2+} oscillations¹¹.

antigen, this external information or first messenger is translated into internal second messengers such as Ca^{2+} , which is a major player in cellular information processing. Cells have access to two sources of signal Ca^{2+} : it can enter from the outside, or it can be released from internal stores using either inositol-1,4,5-trisphosphate (InsP_3) receptors or ryanodine receptors (RZR). One of the primary functions of Ca^{2+} entry is to charge up the internal stores which then release a burst of signal Ca^{2+} responsible for activating a large variety of Ca^{2+} -dependent cellular processes. For example, as Dolmetsch *et al.*² demonstrate, the naive B cells shown in Fig. 1 produce a large-amplitude signal whereas the self-tolerant cells respond to the same external stimulus by producing a much smaller but sustained signal.

The existence of such graded signals introduces a major paradox with regard to Ca^{2+} signalling, because both the InsP_3 receptors and the RZR display a process of Ca^{2+} -induced Ca^{2+} release (CICR) which means that Ca^{2+} signals should be all-or-none and not graded. This paradox is particularly evident in the heart, where the release of Ca^{2+} is based on the positive feedback process of CICR, yet contractions can be graded by varying membrane potential.

The answer to this paradox has emerged recently through exciting new discoveries on the elementary events that constitute typical global Ca^{2+} signals. These elementary events result from the opening of either individual or small groups of channels located on the internal stores^{6,7} (Fig. 2). The events appear to be organized as a hierarchy⁷. The opening of individual channels are fundamental events referred to as 'blips' in the case of the InsP_3 receptors or 'quarks' for the RZR. The next level of organization is represented by small groups of channels (some 10–20) releasing Ca^{2+} as a localized unit to give the 'puffs' and 'sparks' that have been seen in *Xenopus* oocytes and cardiac cells respectively. Work on the heart has uncovered the important principle that these elementary events are autonomous units which can be activated independently of each other, thus providing a neat explanation for the paradox concerning graded Ca^{2+} release (reviewed in ref. 6). Depending on the level of stimulation, the amplitude of Ca^{2+} signals can be graded simply by recruiting a variable number of these elementary events. Whether or not this variable recruitment principle is applicable to the graded Ca^{2+} signals recorded in B cells (Fig. 1) remains to be seen.

In addition to AM and FM, further diversity within the Ca^{2+} signalling system stems from compartmentalization. Earlier this year, it was shown that cytosolic Ca^{2+} signals activate one pathway to gene expression (that mediated by the serum response element) whereas transcription through

another pathway (mediated by the so-called CRE-binding protein) requires a nuclear Ca^{2+} signal⁸. The discovery of elementary events has revealed further examples of how such spatial segregation can increase signal diversity. During the brief periods of channel opening at the sites of elementary events, the local concentration rises to very high levels before it dissipates into the surrounding cytoplasm (Fig. 2). Within the micro domain of the elementary event, the high levels of Ca^{2+} activate certain Ca^{2+} -dependent processes (that is, those that are less sensitive than the processes stimulated by the global signals) such as the activation of ion channels⁹, exocytosis¹⁰ and mitochondrial metabolism¹¹ (Fig. 2).

All in all, the answer to the question of how Ca^{2+} can regulate diverse cellular processes is becoming clearer. Its ability to transmit information is greatly enhanced

through the use of both frequency and amplitude modulation. Further flexibility arises by confining these different signalling modes to discrete cellular locations. □

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1. Rapp, P. E. & Berridge, M. J. *J. Exp. Biol.* **93**, 119–132 (1981).
2. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. & Healy, J. I. *Nature* **386**, 855–858 (1997).
3. Healy, J. I. *et al. Immunity* **6**, 419–428 (1997).
4. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. *Nature* **319**, 600–602 (1986).
5. Gu, X. & Spitzer, N. C. *Nature* **375**, 784–787 (1995).
6. Berridge, M. J. *J. Physiol. (Lond.)* **499**, 291–306 (1997).
7. Bootman, M. D., Niggli, E., Berridge, M. J. & Lipp, P. *J. Physiol. (Lond.)* **499**, 307–314 (1997).
8. Hardingham, G. E., Chawla, S., Johnson, C. M. & Bading, H. *Nature* **385**, 260–265 (1997).
9. Nelson, M. T. *et al. Science* **270**, 633–637 (1995).
10. Tse, F. W., Tse, A., Hille, B., Horstmann, H. & Almers, W. *Neuron* **18**, 121–132 (1997).
11. Hajnoczky, G., Robb-Gaspers, L. D., Seitz, M. B. & Thomas, A. P. *Cell* **82**, 415–424 (1995).

Plasma physics

Magnetic energy release on the Sun

James A. Klimchuk

The Universe abounds with examples of explosive energy release that can heat plasma to extreme temperatures and accelerate particles to relativistic velocities. Such occurrences are not uncommon on our own star, the Sun, where phenomena such as solar flares can reach temperatures of tens or even hundreds of millions of degrees — vastly hotter than the surface of the star. In many cases, a magnetic field is the only source of energy available to power these cosmic explosions. That is certainly true of flares, as well as a host of other less energetic solar phenomena. But whereas the existence of a large reservoir of solar magnetic energy is well established, the reason for its sudden release is still debated.

The most widely accepted explanation is a process known as magnetic reconnection, which effectively involves the cutting and reattachment of magnetic lines of force. Many argue that reconnection has a sound observational and theoretical basis, although it is clear that our understanding is far from complete. Sceptics counter that there is not yet definitive proof that reconnection actually occurs on the Sun. It is for this reason that the new results of Innes *et al.*, reported on page 811 of this issue¹, are so important. They provide the best evidence to date for the existence of bi-directional outflow jets, a fundamental part of the standard reconnection model.

Energy can be extracted from a magnetic field only if the field is stressed. In one common configuration, antiparallel fields are pressed against each other to form a thin sheet of electrical current at their interface. Electrical resistance to this current flow

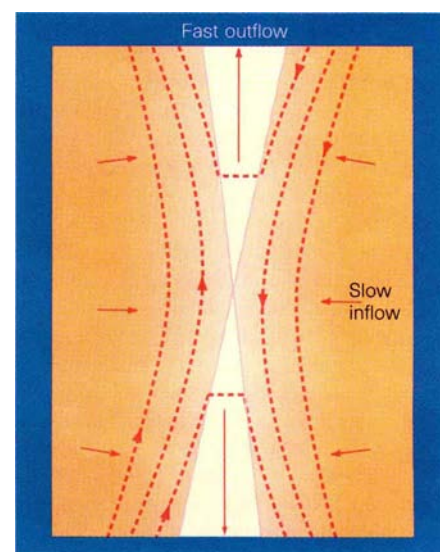


Figure 1 How reconnection makes jets. Plasma flows inwards from each side carrying antiparallel magnetic fields; at the centre of the X, resistive dissipation allows the field to reconnect into sharply curved loops, which catapult the plasma out to the top and bottom. (Adapted from Fig. 2b of Innes *et al.*¹.)

liberates energy from the system to heat the plasma, much as the filament of a light bulb is heated; but because the electrical resistivity of the solar atmosphere is so low, the resistive heating timescale is generally on the order of millennia. Clearly, a more rapid process must be at work.

Enter magnetic reconnection. In 1964, Harry Petschek² made the clever suggestion that resistive dissipation simply facilitates energy release, rather than being the main

responsible agent. Consider Figure 1. Anti-parallel magnetic field lines are carried inward from both sides by a converging flow. The plasma and magnetic field are nearly frozen together due to the low electrical resistivity⁷, and in the simplest geometry, the magnetized plasma tends to pile up at the central sheet and exert a back pressure that opposes further inflow. However, in the modified X-type configuration shown, resistive dissipation allows the field lines to slip through the plasma in a tiny unresolved region at the centre of the 'X'. Field lines entering this so-called diffusion region are effectively cut and reconnected with partner field lines from the opposite side. New field lines are created which are sharply bent, and so experience a strong magnetic tension force that snaps them upward and downward, analogous to the release of a plucked rubber band. Plasma attached to these fields is accelerated to form an opposed pair of collimated outflows, thus evacuating the central region and allowing new magnetized plasma to flow in from the sides.

Note that resistive dissipation at the X-point is energetically unimportant. Nearly all of the magnetic energy release takes place along the extended legs of the X, which are a special kind of stationary shockwave. As inflowing plasma crosses the shock, it is abruptly heated and accelerated at roughly a 90° angle to its original direction. Without the X-point dissipation, however, the field lines could not be cut and reconnected, magnetized plasma would accumulate, and the entire process would break down.

Recent observations have greatly strengthened the case for the existence of magnetic reconnection. The Yohkoh satellite, a joint Japanese-US-UK project, has obtained more than a million X-ray images of the Sun, which are as beautiful⁴ as they are informative. Proceedings from a Yohkoh conference devoted entirely to magnetic reconnection have just been published⁵. But the newest data are now arriving from another highly successful international collaboration, the ESA-NASA Solar and Heliospheric Observatory⁶ (SOHO), and it is in the ultraviolet spectra from SOHO (Fig. 2) that Innes *et al.*¹ saw bi-directional outflow jets. They found redshifted and blueshifted emissions with growing spatial offsets, indicating opposing flows extending to increasing distances from their source. Possible evidence for jets had been noted in earlier spectroscopic data⁷, but the examples were not as clear.

As in all areas of science, the greatest progress is made through a combination of observation and theory. The recent observational advances have been accompanied by equally important theoretical breakthroughs. We are only now beginning to explore the three-dimensional aspects of reconnection, and in the process we are discovering fascinating and fundamental

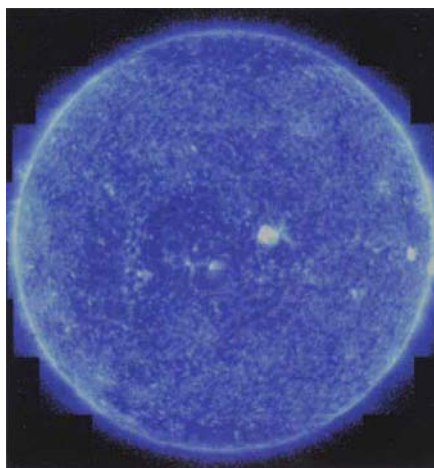


Figure 2 Neon sign. 600,000-kelvin gas on the Sun shows up in ultraviolet emission from highly ionized neon. Spectra from the same instrument (SUMER) have now shown how the gas gets so hot.

effects that could not have been anticipated on the basis of previously restricted two-dimensional views. These include a menagerie of topological complexities^{8,9}, the tunnelling of magnetic flux tubes through each other¹⁰, the formation of extended volumes of filamentary currents¹¹, and the possible inhibition of reconnection by the interlinking of field lines¹². It is worth noting that, even in a two-dimensional context, reconnection may not be as steady and orderly as described above¹³.

Further theoretical progress is being driven by rapid advances in supercomputer technology. Numerical simulation of reconnection is extremely challenging because of

the highly disparate spatial scales involved: resistive diffusion occurs on a very small scale, while equally important flow patterns occur on a very large scale. The difference in scale can exceed 10^{10} . Massively parallel computers and adaptive numerical gridding techniques should make it possible for the first time to adequately differentiate the physical effects of both regimes within a single simulation run. When combined with observations from SOHO, Yohkoh, and planned future missions, it is clear that we are entering a golden era in reconnection studies. □

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1. Innes, D. E., Inhester, B., Axford, W. I. & Wilhelm, K. *Nature* **386**, 811–813 (1997).
2. Petschek, H. E. in *The Physics of Solar Flares* (ed. Hess, W. N.) 425–437 (NASA SP-50, Washington DC, 1964).
3. Jackson, J. D. *Classical Electrodynamics* Sect. 10.3 (Wiley, New York, 1975).
4. Nightingale, L. *Nature* **377**, 105 (1995).
5. Bentley, R. D. & Mariska, J. T. (eds) *Magnetic Reconnection in the Solar Atmosphere* (Conf. Ser. Vol. 111, Astron. Soc. Pacif., San Francisco, 1996).
6. Domingo, V., Fleck, B. & Poland, A. I. *Solar Phys.* **162**, 1–37 (1995).
7. Dere, K. P., Bartoe, J.-D. F., Brueckner, G. E., Ewing, J. & Lund, P. J. *Geophys. Res.* **96**, 9399–9407 (1991).
8. Priest, E. R. & Titov, V. *Phil. Trans. R. Soc. Lond. A* **354**, 2951–2992 (1996).
9. Galsgaard, K. & Nordlund, A. *J. Geophys. Res.* **102**, 231–248 (1997).
10. Dahlburg, R. B., Antiochos, S. K. & Norton, D. *Phys. Rev. E* (in the press).
11. Karpen, J. T., Antiochos, S. K. & DeVore, C. R. *Astrophys. J. Lett.* **460**, L73–L76 (1996).
12. Klimchuk, J. A. in *Magnetic Reconnection in the Solar Atmosphere* (eds Bentley, R. D. & Mariska, J. T.) 319–330 (Conf. Ser. Vol. 111, Astron. Soc. Pacif., San Francisco, 1996).
13. Biskamp, D. *Nonlinear Magnetohydrodynamics* (Cambridge Univ. Press, 1993).

Cancer-susceptibility genes

Gatekeepers and caretakers

Kenneth W. Kinzler and Bert Vogelstein

The identification of cancer-susceptibility genes has revolutionized our understanding of cancer. Most of these genes were originally thought to control cellular proliferation directly, acting as 'gatekeepers'. But in the last few years it has become clear that genes that maintain the integrity of the genome ('caretakers') may be even more frequent causes of inherited predispositions to cancer. Now, on pages 772 and 804 of this issue, Milner *et al.*¹ and Sharan *et al.*² illuminate entirely different properties of the breast-cancer-susceptibility genes *BRCA1* and *BRCA2*, emphasizing the important conceptual and practical differences between gatekeeper and caretaker genes.

Defining the biochemical and biological functions that are relevant to tumorigenesis can be particularly vexing with genes like *BRCA1* and *BRCA2*, which are large and probably have many functional domains. Although *BRCA1* and *BRCA2* are unrelated

at the sequence level, recent research has revealed intriguing similarities between them. First, both contain a region that can act as a transcriptional-activation domain when it is fused to a DNA-binding domain from another gene^{1,3,4}. Naturally occurring mutations found in both *BRCA1* and *BRCA2* in breast-cancer families can compromise this transcriptional activation. As transcription factors are often found among the gatekeeper class of cancer-susceptibility genes, this property indicates that *BRCA1* and *BRCA2* may directly control cellular proliferation. Moreover, *BRCA1* can inhibit the growth of cells in which it is overexpressed⁵, and there is a link between an inhibitor of cell-cycle-dependent kinases and the *BRCA1* protein⁶. However, each of these functional attributes of *BRCA* is of uncertain specificity.

The second similarity is that both *BRCA1* and *BRCA2* bind to Rad51, a protein that is involved in maintaining the integrity of the

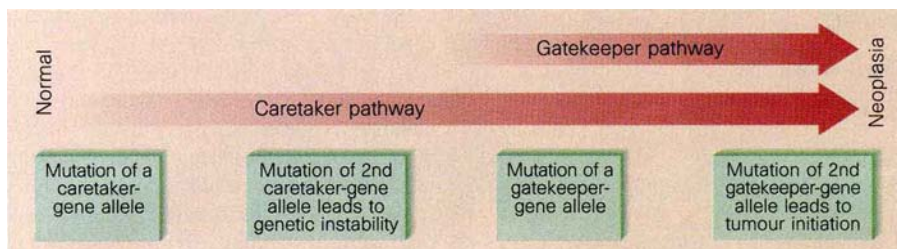


Figure 1 Pathways to neoplasia. Milner *et al.*¹ and Sharan *et al.*² describe different properties of the breast-cancer susceptibility gene *BRCA2*, which could be consistent with its acting as a gatekeeper or a caretaker gene. Inherited mutation of either type of gene can predispose a person to neoplasia, but additional genetic changes are needed to convert a predisposed cell to a neoplastic cell. In the caretaker pathway, three additional mutations are usually required, although the genetic instability that follows inactivation of the second caretaker allele accelerates the accumulation of the latter mutations. In the gatekeeper pathway, only one additional mutation (inactivation of the second gatekeeper allele) is needed to initiate neoplasia. (Although the concepts depicted apply to all inherited cancer susceptibilities, there are variations. For example, inherited mutations of both alleles of a caretaker gene occur in recessively inherited diseases such as xeroderma pigmentosum, and a single dominant-negative mutation can substitute for two inactivation mutations of a gatekeeper gene.)

genome^{2,7}. As well as showing that *BRCA2* binds to Rad51, Sharan *et al.*² report that *Brca2* knockout mice show early embryonic lethality and hypersensitivity to irradiation, similar to that observed in *Rad51* knockout mice. Earlier this year, *BRCA1* was found to bind Rad51 (ref. 7), and *Brca1* knockout mice also show early embryonic lethality^{6,8}. *BRCA1* and Rad51 proteins also share a striking colocalization along synaptonemal complexes (junctions between meiotic chromosomes, necessary for homologous recombination)⁷.

What is the biological consequence of the interaction between the *BRCA* genes and *Rad51*? Human *Rad51* is one of a growing number of eukaryotic homologues of the *Escherichia coli RecA* gene⁹. Studies in both yeast and mammalian cells indicate that *Rad51* is involved in resolving double-stranded DNA breaks and in recombination-linked repair. This is consistent with the observed hypersensitivity to γ -irradiation in embryonic and trophoblast cells from both *Brca2* and *Rad51* knockout mice^{2,10}. So disruption of this *BRCA/Rad51* pathway might be expected to lead to genetic instability. Although the nature of such a putative instability is not clear, studies of Rad51 provide a clue — mouse cells that are deficient for *Rad51* show frequent abnormal mitoses with a markedly reduced chromosome number, suggesting that alterations of this pathway might lead to gross chromosomal changes¹⁰.

Independent lines of investigation have therefore suggested two disparate, yet intriguing, functions for the *BRCA* genes. But which of these functions is most likely to underlie their true role in tumorigenesis? We believe that the relationship between inherited and non-inherited ('sporadic') forms of cancer provides additional information which leads to the conclusion that the *BRCA* genes are caretakers rather than gatekeepers.

Gatekeepers are genes that directly regulate the growth of tumours by inhibiting growth or promoting death. Each cell type has only one (or a few) gatekeepers, and inactivation of a given gatekeeper leads to a very specific tissue distribution of cancer. For example, inherited mutations of the *Retinoblastoma*, *von Hippel Lindau*, *Neurofibromatosis type 1* and *Adenomatous polyposis coli* genes lead to tumours of the retina, kidney, Schwann cells and colon, respectively. Inactivation of these genes is rate-limiting for the initiation of a tumour, and both the maternal and paternal copies must be altered for tumour development (Fig. 1). Predisposed individuals inherit one mutant copy of the gatekeeper gene, so they need only one additional (somatic) mutation to initiate neoplasia. Sporadic tumours form in people who do not have a germline mutation when both copies of the relevant gatekeeper gene become mutated somatically. Because the probability of acquiring a single somatic mutation is exponentially greater than the probability of acquiring two such mutations, people with a hereditary mutation of a gatekeeper gene are at a much greater risk ($>10^3$) of developing tumours than the general population. This is the essence of Knudson's hypothesis, which has guided the field for the past 20 years (reviewed in ref. 11).

In contrast, inactivation of a caretaker gene does not promote tumour initiation directly. Rather, neoplasia occurs indirectly — inactivation leads to genetic instabilities which result in increased mutation of all genes, including gatekeepers. Once such a tumour is initiated by inactivation of a gatekeeper gene, it may progress rapidly due to an accelerated rate of mutation in other genes that directly control cell birth or death.

In dominantly inherited cancer-predisposition syndromes of the caretaker type, patients inherit a single mutant caretaker gene from an affected parent. Three sub-

sequent somatic mutations are usually required to initiate cancer: mutation of the normal caretaker allele inherited from the unaffected parent, followed by mutation of both alleles of a gatekeeper gene. Because three mutations are needed, the risk of cancer in affected families is generally only 5–50-fold greater than in the general population — much less than the risk in families with inherited defects in a gatekeeper gene¹². Moreover, mutations in caretaker genes would not be expected to lead to sporadic cancers very often, as four mutations would be required (two caretaker alleles plus two gatekeeper alleles).

Known caretaker genes include the nucleotide-excision-repair genes that are responsible for xeroderma pigmentosum, the mismatch-repair genes that cause hereditary nonpolyposis colorectal cancer, and probably the *ATM* gene, which is responsible for ataxia telangiectasia. To this list should probably now be added *BRCA1* and *BRCA2*. Consistent with this hypothesis, mutations in *BRCA1* and *BRCA2* are rarely found in sporadic cancers, and the risk of cancer arising in people with *BRCA* mutations is relatively low. The data from Sharan *et al.*² and Scully *et al.*⁷, relating *BRCA1* and *BRCA2* to *Rad51*, provide a biochemical explanation that supports the hypothesis.

The distinction between gatekeepers and caretakers has important practical, as well as theoretical, ramifications. In particular, tumours that have defective caretaker genes present an additional therapeutic target. Such tumours would be expected to respond favourably to therapeutic agents which induce the type of genomic damage that is normally detected or repaired by the particular caretaker gene involved. The discovery by Sharan *et al.*² — that mouse cells with defective *Brca2* genes are sensitive to γ -irradiation — is therefore particularly noteworthy. It indicates that tumours from breast-cancer patients with inherited *BRCA* mutations should be more sensitive to such radiation than other breast cancers. This hypothesis can easily be tested and, if borne out by experiment, it would have obvious and substantial implications for treatment. □

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1. Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M. & Kouzarides, T. *Nature* **386**, 772–773 (1997).
2. Sharan, S. K. *et al.* *Nature* **386**, 804–810 (1997).
3. Chapman, M. S. & Verman, I. M. *Nature* **382**, 678–679 (1996).
4. Monteiro, A. N., August, A. & Hanafusa, H. *Proc. Natl. Acad. Sci. USA* **93**, 13595–13599 (1996).
5. Holt, J. T. *et al.* *Nature Genet.* **12**, 298–302 (1996).
6. Hakem, R. *et al.* *Cell* **85**, 1009–1023 (1996).
7. Scully, R. *et al.* *Cell* **88**, 265–275 (1997).
8. Liu, C.-Y. *et al.* *Genes Dev.* **10**, 1835–1843 (1996).
9. Heyer, W.-D. *Experientia* **50**, 223–233 (1994).
10. Lim, D. S. & Hasty, P. *Mol. Cell Biol.* **16**, 7133–7143 (1996).
11. Knudson, A. G. *J. Cancer Res. Clin. Oncol.* **122**, 135–140 (1996).
12. Kinzler, K. W. & Vogelstein, B. *Cell* **87**, 159–170 (1996).

Really ancient DNA

Lights turning red on amber

Bryan Sykes

Were science to be judged on entertainment value alone, there is little doubt that the fastest route to the glittering prizes would be to work on fossil DNA. If you doubt that, take a look at the British tabloids of 9 March, where a report linking the DNA from a Palaeolithic tooth to a twentieth-century schoolteacher got the full treatment which, for one paper, meant two 'topless models posing with hastily assembled flint axes. Very little captures the public imagination quite so much as recovering the DNA from our ancestors and other extinct organisms, with the unspoken promise of re-creating them. Nowhere was this better realized, of course, than in the film *Jurassic Park*. An excellent film in many ways, but whose appeal was surely enhanced by reports that the basic scientific premise — that DNA could survive for millions of years, especially when protected by amber — was essentially correct. The findings were widely reported in the scientific press which, like the tabloids (but with a restrained repertoire for pictorial embellishment), know a good story when they see one.

IMAGE
UNAVAILABLE
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REASONS

Figure 1 A fly in the ointment for those who believe that DNA can survive for millions of years.

So are the stories about amber true? A paper by Austin *et al.*¹, published this week in *Proceedings of the Royal Society*, comes up with a resounding "no". In an exhaustive series of experiments using insects trapped in Oligocene Dominican amber, as well as insects

in the much younger Quaternary East African copal (a resin from tropical trees), they have been unable to find any traces of credibly ancient DNA in the sort of specimens that have been reported as being abundant sources of the real thing. The authors examined more specimens than all six of the previous studies combined — studies that had reported success in 13 out of 14 cases. Although Austin *et al.* could not reproduce the results because the individual samples were, of course, different, the findings nonetheless cast doubt on the original reports.

What's to be made of the claims for the revival of dead DNA after several million years? In one case — the celebrated retrieval of DNA from an unidentified Cretaceous dinosaur fossil (not in amber, I hasten to add) — the reported sequences² were almost certainly from nuclear inserts derived from human mitochondria³. In another study, a 20-million-year old magnolia leaf produced sequences that were enticingly like modern magnolias⁴. When the derived sequences are similar, but not identical, to those of living relatives, there has been an understandable temptation to use this as evidence of authenticity for the ancient DNA, through phylogenetic affinity blurred by time. But it now seems clear, from the accumulated experience of a number of laboratories, that when the polymerase chain reaction (PCR) is used to amplify minute amounts of damaged template, it can create bizarre amplicons (amplified sequences) from low-level modern contaminant DNA, the sequence of which has been scrambled by 'jumping' PCR⁵.

Both leaves and dinosaurs were fully exposed to water and oxygen, the agents which, through the inexorable processes of hydrolysis and oxidation, set a theoretical upper limit of 50,000 to 100,000 years, beyond which no amplifiable material would survive^{6,7}. Only amber — in which entombed specimens are completely dehydrated — had the potential to thwart this logic. Studies of levels of the D and L enantiomers of aspartic acid from amber-entrapped insects raised hopes that this might be the case⁸. They showed that the normal diagenetic process that would equilibrate these two optical isomers after a million years or so under normal hydrated conditions, and which seemed to correlate with the ability to recover endogenous DNA, had apparently been halted. However, Austin *et al.*¹ have effectively snuffed out this glimmer of optimism.

Where does this leave the original reports? For a start, they have not crossed the hurdle of independent verification. But whether these reports owe their existence to serendipity or to overenthusiastic interpretation, I can't help feeling a tinge of disappointment that the sober and meticulous efforts of Austin *et al.* have ended the way that they have. Spoilsports? Yes, a little, but

Exhibition

Not from books but from dissections

Beware the dangers of library research. This *Skeleton kneeling in prayer before a shelf of books* (by Jaques Gamelin, 1779) has clearly been overdoing it. Perhaps he should get out more, and go to a few exhibitions. *The Ingenious Machine of Nature: Four Centuries of Art and Anatomy* runs from 19 April to 15 June at the Philadelphia Museum of Art, with more than 100 works by artists from Dürer to Stubbs, following European scientific anatomy from its beginnings in sixteenth century Italy. These drawings were not only essential for anatomists (Leonardo Da Vinci was scornful of using words to understand the body: "the more thoroughly you describe the more you will confuse"), they also became invaluable in the instruction of other artists.

Stephen Battersby

IMAGE
UNAVAILABLE
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REASONS

now the ball is firmly back in the court of the original protagonists, who must in future be more prepared than hitherto to share whatever samples they are claiming to be authentic. Although Austin *et al.* have a point when they conclude that "the primary value of amber-preserved fossils lies in their excellent morphological preservation and not in the fragmented remains of any DNA whose existence remains speculative at best", I, for one, hope that the authors of the original reports will rise to the challenge. □

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1. Austin, J. J., Ross, A. J., Smith, A. B., Fortey, R. A. & Thomas, R. H. *Proc. R. Soc. Lond. B* **264**, 467–474 (1997).
2. Woodward, S. R., Weyand, N. J. & Bunnell, M. *Science* **266**, 1229–1232 (1994).
3. Zischler, H. *et al. Science* **268**, 1192–1193 (1995).
4. Golenberg, E. M. *et al. Nature* **344**, 656–658 (1990).
5. Pääbo, S., Irwin, D. M. & Wilson, A. C. *J. Biol. Chem.* **265**, 4718–4721 (1990).
6. Lindahl, T. *Nature* **365**, 700 (1993).
7. Sykes, B. *Nature* **366**, 513 (1993).
8. Poinar, H. *et al. Science* **272**, 864–866 (1996).

Planetary exploration

Sighting the seas of Europa

William B. McKinnon

Europa is the second of the galilean satellites out from Jupiter. Since the Voyager missions in 1979, an ocean has been suspected under its covering of ice (ref. 1, for example). For some, even the possibility of liquid water elsewhere in the Solar System brings fevered dreams of extraterrestrial life, so new images and data from Europa, transmitted from the Galileo spacecraft now orbiting Jupiter, have been eagerly awaited. The spacecraft has made two close passes by the satellite, on 19 December 1996 and 20 February 1997, and many of the new data were presented for the first time at a conference last month*. Europa stands revealed as a dynamic and geologically active satellite, and there now appears to be direct evidence for subsurface water. But the picture is complicated, and that we cannot yet say whether a global ocean exists.

Europa's trek from planetary backwater to potential exobiological centre-stage has been a long one. After the Voyager missions, and the realization of the importance of tidal heating in satellite evolution, theoretical models appeared that allowed the possibility of a liquid layer underneath the surface ice shell and above the rocky interior. As the density of the 1,570-km-radius body is very close to 3.0 g cm⁻³, the bulk of Europa must be composed of rock and metal, and estimates of the thickness of ice and/or water ranged from a few kilometres to nearly 200. Any liquid layer would thus be thin compared with the satellite's radius, and near the surface, qualifying as the Solar System's second ocean. The amount of tidal heating within Europa is, however, much less than within fiendishly volcanic Io, the innermost galilean moon, so it has not been possible to establish with certainty that Europa must possess an ocean, based on theory alone. A well-known series of papers, mostly by the same authors, supported, then denied, then supported, and then equivocated on the likelihood of a european ocean^{1–4}.

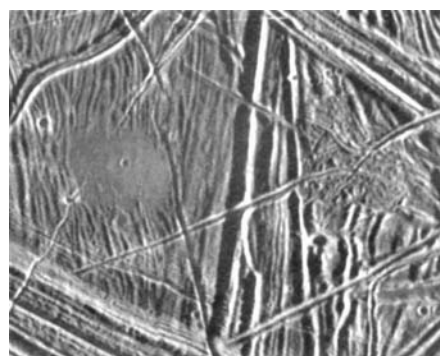


Figure 1 A 3-km-wide patch where liquid water erupted and froze on the european surface. Sun is from right.

Voyager images of Europa were of much lower resolution and coverage than for the other galilean satellites, but provided some tantalizing clues. From them, P. M. Schenk first argued that blocks of Europa's icy crust had separated and rotated past one another, and that these blocks or plates could be fitted into older patterns, indicating a sort of incipient plate tectonics. This work was regarded with some scepticism at the time (the early 1980s), but eventually passed peer-review muster⁵.

A long-range image of Europa taken last November⁶ has now provided clear and

concrete proof of this interpretation. The wedge-shaped regions of separation between the plates are built of parallel sets of linear structures that resemble the separation and infilling at the Earth's mid-ocean ridges. More significantly, a detailed computer-aided reconstruction of the image (R. Tufts, Univ. Arizona) now shows that one can work back through three stages of rotation and spreading in that 250-km-wide region. Large areas of Europa imaged earlier in the mission⁷ can be similarly reconstructed (R. Sullivan, Arizona State Univ.).

None of this absolutely requires liquid water; the shifting of brittle, icy plates could occur on softer (presumably hotter), deep ice. The global system of linear features identified by Voyager offers a potentially more powerful, albeit more subtle, indication of water. The best explanation for the geometric pattern of these lineaments is that they form as tension or extension fractures while Europa rotates slightly faster than synchronously — that is, not keeping the same face to Jupiter. Stresses arise because the icy shell must conform to the triaxial, tidally distorted shape of the satellite as the shell moves with respect to the tidal axis, which is elongated towards Jupiter by about 0.5 km (R. Greenberg, Univ. Arizona).

Such nonsynchronous rotation had originally been proposed as a consequence of a supposed lack of permanent mass asymmetry within a dominantly fluid Europa⁸, a sufficiently large asymmetry being necessary for the jovian tides to secure a purchase on Europa and hold it to a synchronous spin. Our geophysical experience with silicate bodies, however, even geologically active ones, suggests that the rocky interior of Europa should possess a large enough mass asymmetry to be tidally locked. So if the icy surface has rotated nonsynchronously, it must have slipped relative to the rock interior, which all but demands decoupling by liquid water — *et voilà, une mer*.

The higher resolution of the Galileo images now allows the global lineament pattern to be sorted by age (P. Geissler, Univ. Arizona). Three distinct stages of lineament

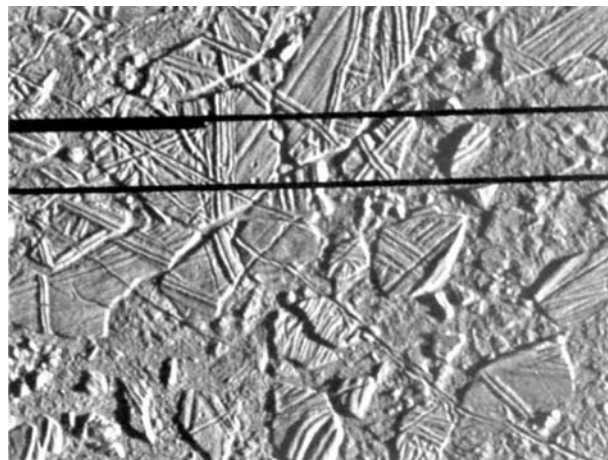


Figure 2 Pack ice and icebergs on Europa? This chaotic scene is 42 km across. Sun is from right.

* Lunar and Planetary Science XXVIII, Houston, Texas, USA, 15–19 March 1997.

formation are seen over one broad region, and are consistent with a tidal stress pattern that has moved or migrated from east to west, or, equivalently, with an icy shell that is moving from west to east with respect to the tidal axis — that is, faster than synchronously.

The european surface is young (C. R. Chapman, Southwest Research Inst., Boulder). The relative lack of craters more than 10 km in diameter, inferred from Voyager, is confirmed; and even craters about 100 m across, which are generally scarce on the galilean satellites, are all but absent on some parts of Europa. Europa must have been resurfaced over some period, but when? That is uncertain, because the absolute flux of impacting comets and asteroids is not well known, but an overall surface age of less than 10 million years is possible^{9,10}. This age is intriguing, as the most detailed theoretical model of the tidal heating of a free-floating ice shell¹¹ predicts just this rotation period for the nonsynchronous rotation of the shell. That is the time it takes a thermal wave to propagate through the shell so that the thickness of the shell can adjust to a tidal heating input that varies with position with respect to the tidal axis.

But what do the new images, including those released just last week, have to say directly about the presence of subsurface water? (T. V. Johnson, JPL; R. Greeley, Arizona State Univ. and others) Figure 1, from the December encounter, shows a smooth patch only 3 km wide. It is quite flat, and appears to bury a set of ridges and grooves. No raised flow margins are seen, which would have indicated its emplacement as a viscous slurry or 'hot' glacial ice. The simplest interpretation is that this indeed records the eruption of liquid water, possibly with other components, onto the surface. Similar examples can be seen in other pictures, as can many different eruptive styles, such as obviously viscous extrusions. In other places the surface has buckled upward and simply fractured, without any volcanism. In yet other cases the surface appears dimpled or depressed. In the example in Fig. 1, the erupted water may have been contained in such a depression.

Figure 2 is an even more spectacular image, taken in February, of disrupted terrain on the trailing hemisphere of the satellite. A region of ridges and grooves has fragmented into a series of blocks, up to about 10 km across. These blocks have moved and rotated with respect to one another, and are set in a 'sea' of lower-lying hummocky material. Several of the blocks appear tilted and partially submerged. The overall appearance is, without exaggeration, reminiscent of terrestrial pack-ice disintegrating in the spring. Similar regions may exist throughout Europa's so-called 'mottled terrain' (T. V. Johnson, personal communication). Again, liquid water, ice-encrusted in this case, appears to have

reached and/or replaced the surface.

What is most astonishing about Fig. 2, however, is not the disruption itself, but the degree and distribution of heating that would have been necessary to create such melting and chaos. The thermal pulse required exceeds the tidal heating calculated in all the models published to date. It is also doubtful whether even a very active region of volcanism in the underlying rock¹² could so effectively channel its heat flux to an icy shell that would be separated by about 100 km of water, in the standard ice/ocean/rocky-core structure. Perhaps the ice shell is actually much thinner, and coupled directly to the rocky core (L. Wilson, Univ. Lancaster). Gravity data from Galileo should ultimately decide whether the ice/water layer is thick or thin, but at this point, even with excellent evidence for the recent presence of subsurface water on Europa, we cannot say whether this water was local or globally connected into an ocean or oceans, or even if it is still there.

The chaotic region does, however, mean that such an ocean may be accessible to further exploration. The blocks or rafts in Fig. 2 are probably no more than a few kilometres thick, otherwise they would not have fragmented as they have. On the other hand, a new, high-resolution image of the fresh, 26-km-wide crater Pwyll, some 1,000 km away, shows no obvious sign that the impact punched through or fractured such a thin ice crust. The ice thickness

may be greater than 10 km (more in keeping with tidal heating models¹³).

In order to map the ice thickness, and to determine whether the ice layer is decoupled from the rocky interior by an ocean, new types of data will be needed. NASA is now studying the possibility of sending an orbiter to Europa with a long-wavelength radar sounder to tackle the former question, and a laser altimeter to measure tidal flexing and deal with the latter. Although the existence of life on Europa is still speculative, the scientific opportunity offered by this satellite is a tide that surely must be taken at the flood. □

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1. Cassen, P., Reynolds, R. T. & Peale, S. J. *Geophys. Res. Lett.* **6**, 731–734 (1979).
2. Cassen, P., Peale, S. J. & Reynolds, R. T. *Geophys. Res. Lett.* **7**, 987–988 (1980).
3. Squyres, S. W., Reynolds, R. T., Cassen, P. M. & Peale, S. J. *Nature* **301**, 225–226 (1983).
4. Ross, M. N. & Schubert, B. *Nature* **325**, 133–135 (1987).
5. Schenk, P. M. & McKinnon, W. B. *Icarus* **79**, 75–100 (1989).
6. Kerr, R. A. *Science* **274**, 2015 (1996).
7. Belton, M. J. S. *et al. Science* **274**, 377–391 (1996).
8. Greenberg, R. & Weidenschilling, S. J. *Icarus* **58**, 186–196 (1984).
9. Shoemaker, E. M. *et al. Lunar Planet. Sci. XVII*, 799–800 (1986).
10. Shoemaker, E. M. *Europa Ocean Conf. Abstr.* 65–66 (San Juan Capistrano Res. Inst., 1996).
11. Ojakangas, G. W. & Stevenson, D. J. *Icarus* **81**, 242–270 (1989).
12. McKinnon, W. B. *Europa Ocean Conf. Abstr.* 50–51 (San Juan Capistrano Res. Inst., 1996).
13. Ojakangas, G. W. & Stevenson, D. J. *Icarus* **81**, 220–241 (1989).

Human genetics

Breaking the rule of three

Jean-Louis Mandel

Since 1991, at least a dozen human genetic diseases have been shown to be caused by unstable expansions of trinucleotide repeats¹ (Fig. 1, overleaf). The discovery of these expansions was a surprise because they had not been described in other organisms, but it led to a tendency to derive general rules based on the available observations. These rules may now need to be reconsidered with the publication of a report by Laloti *et al.*² on page 847 of this issue and two papers in *Nature Genetics*^{3,4}, which show that the expansion of a larger repeat unit is responsible for a monogenic form of epilepsy.

Above a threshold of about 35–50 repeats (100–150 base pairs), trinucleotide repeats that are linked to diseases become unstable, and they tend to expand further in successive generations. The length of the repeat correlates with the age of onset of the disease (or in the case of fragile X syndrome, the risk of having affected children), and the tendency to expand depends strongly on the sex of the transmitting parent. The various diseases can be divided into two main classes (Table 1, overleaf). Class 1 diseases are caused by massive expansions in non-coding regions of a

gene: these expansions affect the expression of the gene in both neural and non-neural tissues. Class 2 diseases are caused by moderate expansions of CAG repeats, which code for polyglutamines. Class 2 expansions lead to the synthesis of a toxic protein and the progressive death of specific neuronal populations.

Until the end of 1995, it seemed that the mutations that led to expansion were the hallmark of diseases with anticipation (increased precocity, severity or penetrance in successive generations). It was also thought that only (G+C)-rich repeats (for example, CAG/CTG or CGG/CCG) were implicated, as they could form stable hairpin loops which might interfere with normal DNA replication, thereby accounting for the expansion bias⁵. But then, in 1996, a GAA expansion was discovered in the autosomal recessive disease Friedreich's ataxia⁶. Anticipation cannot be seen in autosomal recessive diseases, because they usually affect only a single generation. Moreover, the GAA repeat cannot form hydrogen-bonded hairpins, although it may be involved in the formation of other, non-conventional DNA structures. More recently⁷, 'premutation'

NATURE

100 YEARS AGO

The influence of music upon the respiration, the heart, and the capillary circulation is the subject of a paper, by MM. A. Binet and J. Courtier, in the *Revue Scientifique* (February 27). Experiments were made upon a well-known musical composer, and the investigators endeavoured to determine effects produced by musical sound alone, as distinct from those due to emotions aroused by pieces associated with dramatic incidents or words.... Both major chords struck in a lively manner and discords quickened the respiration, the latter more especially. Minor chords retarded respiration. When melodies were tried it was found that all, whether grave or gay, produced quickened respiration and increased action of the heart. The lively tunes produced the greatest acceleration. Where the sound was wholly uncomplicated by emotional ideas, as in single notes or chords, the heart's action was accelerated, but not in so marked a degree as when a melody either grave or gay was played. During operatic pieces, or those well known to the subject, the acceleration attained its maximum. ... capillary tracings showed that a slight diminution of pulsation was usually produced by musical sounds, the effect being very small when sad melodies were played, but well-marked when lively airs were played.

From *Nature* 22 April 1897.

50 YEARS AGO

The much-heralded film "The Beginning or the End?" (Metro-Goldwyn-Mayer) was shown privately to a distinguished audience in the Palace Theatre, London, on April 22. Judged by any standard or from any point of view, it is not a good film.... From the documentary point of view it is disappointing, since it really tells so little about all the atomic research which led eventually to the release of atomic energy.... lip-service was paid to the contributions of such geniuses as Rutherford, Joliot, Chadwick, Hahn and a few others, in one or two sentences uttered from his arm-chair by an actor representing Einstein, but whose elocution was so poor that even this small concession will be lost on a lay audience. Meitner, Oliphant, Blackett, Cockcroft, Bohr and other important persons were not mentioned at all.

From *Nature* 26 April 1947.

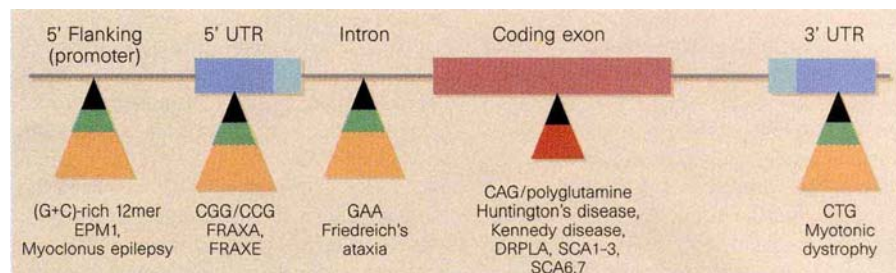


Figure 1 Lalioti *et al.*² have shown that the expansion of a 12-bp repeat is responsible for a monogenic form of epilepsy known as EPM1. This joins a growing number of genetic diseases that are associated with unstable repeat expansions, and the position of the expansion in each case is shown. For the large expansions, black regions represent the number of repeats in the normal range; green areas indicate expansions corresponding to unstable premutations; and the orange regions represent larger expansions that lead to disease. For moderate expansions of the CAG/polyglutamine type, the black area corresponds to normal repeats whereas expansions in the red area lead to disease. For abbreviations, see Table 1.

alleles of around 40–60 GAAs have been seen in Friedreich's ataxia. These can expand in a single generation to pathological length (>200 GAA) — similar to the premutation alleles that are found in the fragile X syndrome and myotonic dystrophy.

The new studies^{2–4} have looked at progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1). This is a rare autosomal recessive disease that is most common in Finland and the western Mediterranean. The gene for EPM1 was mapped to chromosome 21, and the presence of a shared haplotype in Finnish patients allowed it to be identified: it encodes the proteinase inhibitor cystatin B (ref. 8). But only a minority of patients with EPM1 have point mutations in this gene and, in particular, the mutation in the Finnish patients could not be found.

As decreased levels of cystatin B messen-

ger RNA were observed in lymphoblastoid cells from patients with EPM1, several groups began to analyse the promoter region using Southern blotting and the polymerase chain reaction. In the March issue of *Nature Genetics*, Lafrenière *et al.*³ described the presence of a large insertion (600–900 bp) in most patients. The insertion mapped to the site of a tandem repeat of the 12-bp sequence CCCC GCCCGCG, which is repeated two or three times in normal alleles of the gene. Lalioti *et al.*² now report that they have sequenced plasmids that were cloned from both a French and a Swiss patient, and they find that the expansion is made up of an apparently pure repeat of the 12-bp motif.

Lalioti *et al.* have found that in two related non-EPM1 families, the parents have premutation alleles with 12 or 13 repeats. Although the number of transmissions studied is still

Table 1 Disease associated with repeat unstable expansions

Class 1: Massive non-coding expansions			
Repeat motif	Disease	Transmission*	Protein/localization of repeat
CGG (methylated)	Fragile X mental retardation syndrome	XD	FMR1 (RNA binding protein)/5' untranslated region
CCG (methylated)	Fragile X E mild mental retardation	XR	FMR2 (transcription factor?)/5' untranslated region?
CTG	Myotonic dystrophy	AD	DMPK (serine/threonine kinase)/3' untranslated region DMAHP (homeoprotein)/5' flanking
GAA	Friedreich's ataxia	AR	Frxataxin/intronic
(G+C)-rich dodecamer?	Progressive myoclonus epilepsy (EPM1)	AR	Cystatin B (proteinase inhibitor)/5' flanking
Class 2: Moderate CAG/polyglutamine expansion			
Disease	Transmission	Protein	
Spinobulbar muscular atrophy (Kennedy disease)	XR	Androgen receptor	
Huntington's disease	AD	Huntingtin	
Dentatorubropallidoluysian atrophy (DRPLA)	AD	Atrophin	
Spinocerebellar ataxia (SCA1)	AD	Ataxin 1	
SCA2	AD	Ataxin 2	
SCA3	AD	Ataxin 3	
SCA6	AD	α1A calcium channel	
SCA7 (with macular degeneration)	AD	Not cloned	

* Transmissions are X-linked (X) or autosomal (A), and dominant (D) or recessive (A). The large expansions associated with the fragile sites FRAXF, FRA11B, FRA16A and FRA16B have been omitted, as they do not seem to directly cause a disease. With the exception of myotonic dystrophy, the class 1 expansions cause a loss of function (decreased or no transcription) that can be mimicked by conventional mutations in the target genes.

small, the authors found that when these alleles were transmitted maternally they were stable, whereas six out of eight paternal transmissions resulted in expansions of one to four repeats. There were no repeat contractions. Three of the offspring carried two alleles in the 12–17-repeat range, yet they did not show symptoms of EPM1, indicating that these represent premutations. Interestingly, the size of such unstable alleles (144–156 bp) is equivalent to the stability threshold of the trinucleotide repeats.

There is some disagreement as to the exact sequence of the expanded repeat because, in the April issue of *Nature Genetics*, Virtaneva *et al.*⁴ report that they have sequenced a single expanded repeat that has been cloned in bacteriophage lambda. They find an 18-bp repeat at the 5' end of the sequence and a 15-mer at the 3' end — but no evidence for the expansion of a 12-bp motif. Moreover, the 18- and 15-bp sequences contain six and four Ts or As, respectively, so they are probably only distantly related to the 12-bp sequence described by Laloti *et al.*, which contains neither base.

Why is there such a discrepancy between the findings of the two groups? Both reported that it was very difficult to sequence through the repeat and that special conditions were needed, so compression artefacts may have been generated. Another factor may be instability during cloning — the expansion that was partially sequenced by Virtaneva *et al.* was 1 kilobase long in the patient's DNA, but only 400 bp in the lambda clone. A third, but unlikely, possibility is that there is a difference between expansions in different populations. Although both groups studied flanking polymorphic markers, they were unable to say whether the expansions had more than one origin. The discrepancy concerning the nature of the repeat should be resolved quickly, as it is important when considering mechanisms. Instability of trinucleotide repeats (which is thought to arise during DNA replication) requires that all the repeats are the same, whereas sequence heterogeneity is frequent in minisatellite arrays, which are thought to mutate mainly by recombination⁹.

All three reports did agree, however, that the expanded alleles seem to be quite stable within families, and they show no obvious somatic heterogeneity (although Southern blotting is not very sensitive). This is in contrast to similar-sized repeat lengths found in the fragile X syndrome and myotonic dystrophy¹. If Laloti *et al.* are right, the expansion in both EPM1 and fragile X is made up of Gs and Cs only, and results in decreased transcription. But whereas in fragile X the transcriptional shut-off is correlated with abnormal methylation of the expanded region and neighbouring promoter sequence, the methylation pattern seems to be unaffected in EPM1. Moreover, large expansions only occur when fragile X is transmitted maternal-

ly, whereas Laloti *et al.* suggest that EPM1 is unstable following paternal transmission.

The new studies extend the scope of expansion diseases by showing that longer repeat units are involved. An even longer unit (an (A+T)-rich sequence of 33 bp) has recently been shown to be expanded to 15–60 kb in a distamycin A-sensitive fragile site on chromosome 16 (ref. 10), although this does not cause disease. So the rule of three is well and truly dead — although the newly identified repeat units are all multiples of three. All of the known expansion diseases are neurological in the broad sense, but, because such mutations can lead to a loss of function, they may be found linked to any kind of phenotype, and they do not necessarily account for all, or even most, of the mutations in a target disease. Indeed, for several diseases (such as

the X-linked Alport syndrome or ocular albinism), many patients do not show point mutations in the exons of the affected genes^{11,12}, so these may be good loci in which to search for expansions of any kind. □

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1. Paulson, H. L. & Fischbeck, K. H. *Annu. Rev. Neurosci.* **19**, 79–107 (1996).
2. Laloti, M. D. *et al. Nature* **386**, 847–851 (1997).
3. Lafrenière, R. G. *et al. Nature Genet.* **15**, 298–302 (1997).
4. Virtaneva, K. *et al. Nature Genet.* **15**, 393–396 (1997).
5. Wells, R. D. J. *Biol. Chem.* **271**, 2875–2878 (1996).
6. Campuzano, V. *et al. Science* **271**, 1423–1427 (1996).
7. Cossée, M. *et al. Proc. Natl Acad. Sci. USA* (in the press).
8. Pennacchio, L. A. *et al. Science* **271**, 1731–1734 (1996).
9. Buard, J. & Jeffreys, A. J. *Nature Genet.* **15**, 327–328 (1997).
10. Yu, S. *et al. Cell* **88**, 367–374 (1997).
11. Knebelmann, B. *et al. Am. J. Hum. Genet.* **59**, 1221–1232 (1996).
12. Schiaffino, M. V. *et al. Hum. Mol. Genet.* **4**, 2319–2325 (1995).

Neuropsychology

Towards a neuropathology of emotion and mood

Antonio R. Damasio

Depression is one of the prevalent causes of health-related human suffering and is, first and foremost, a disorder of one of the least-studied biological phenomena: emotion. To manage depression effectively, we need to understand the neurobiology of emotion along its many dimensions — molecular, cellular, micro- and macro-system, cognitive and sociocultural — and to apply that understanding to well-characterized disease phenotypes and genotypes. Some progress has been made towards that goal. For example, it is now clear that depression can have a unipolar course (in which it is not accompanied by mania), or a bipolar course (in which depression and mania alternate). We also know that depression can be transmitted genetically, and that selected autonomic, endocrine and neurotransmitter disturbances are part of the biological state that defines depression and mania. Finally, it seems that dysfunction in some brain regions more than others, for instance in the prefrontal cortices, is especially related to either depression or to mania.

Not surprisingly, these developments do not yet amount to a comprehensive view of the problem: that is why the contribution by Drevets and colleagues¹ on page 824 of this issue is so welcome. In a well-planned study that is both careful and persuasive, the authors use a functional imaging method (positron emission tomography, or PET), to show that a relatively well-defined region of the prefrontal cortex, found just under the genu of the corpus callosum (Fig. 1, overleaf), is consistently underactivated in familial depressive patients — both unipolar and bipolar. Moreover, this

same region is *activated* during periods of mania. In a follow-up study based on a structural magnetic resonance imaging (MRI) estimate of mean grey-matter volume, Drevets *et al.* show that the size of this target region is consistently reduced in the left cerebral hemisphere of depressive patients, but not in that of controls.

The functional (PET) imaging result delineates, at a smaller scale than has previously been available, a neural region that is part of the multiplex circuitry of emotion; this region is critically involved in the dysfunctional states of depression and mania. The structural (MRI) result, on the other hand, allows us to ask how the reduced size of the target area in depressive patients might have arisen. Was it determined by the genome at the outset, by genomic and developmental interactions, by the insult resulting from repeated episodes of disease, or by some combination of these mechanisms?

As I see it, then, adopting a 'systems' perspective, Drevets *et al.* have found consistent functional and structural anomalies in one component of a system whose large-scale function is to organize emotional responses to complex personal and social situations. In other words, they have identified a key player in one of the several systems that underlie emotional processing — a valuable finding indeed.

How does malfunction in that system produce such dire results? The stereotaxic coordinates of the region identified by Drevets *et al.* indicate that it sits within the subcallosal portion of Brodmann field 24. Field 25, which is just ventral to field 24, may also be included, as well as a part of field 32.

Neuroanatomical work indicates that field 24 receives, both directly and indirectly, signals from many higher-order association cortices. Field 24 also projects massively to field 25 and to the basal ganglia. Fields 24 and 25 project to the periaqueductal grey, and both are richly interconnected with somatosensory structures (via the insula) and with the amygdala. Moreover, field 25 projects abundantly to the hypothalamus (see ref. 2 for overall review, and refs 3–7).

Given its strategic position, processing in the target region can be affected by activity in the varied neural sectors that collectively support the images that constitute thought. In turn, processing in the target region can affect activity in the neural sectors that produce autonomic, neurotransmitter, endocrine, motor and somatosensory responses, which together enact the phenomenon we call emotion. Finally, the myriad neurochemical and somatomotor parameter changes which embody the emotional state are read out as feelings and, when sustained, as moods. The anomalies identified by Drevets *et al.*¹ probably lead to abnormal moods because the emotional responses that such anomalies help to engender are disproportionately large relative to the thoughts that induce those responses (the anomalies may even give rise to spontaneous, unmotivated, emotional responses), and because such emotional responses are iterated for long periods of time. So they produce a sustained, rather than a brief, change in biological state.

Several other findings, obtained in different clinical and experimental settings, are compatible with the new result, and they lend it additional significance. First, as Drevets *et al.* indicate, the overall region identified in their study has been implicated in the modulation of neurotransmitters such as serotonin, noradrenaline and dopamine, whose levels are manipulated by antidepressant drugs. Second, lesions that encompass the target region in the ventromedial prefrontal cortex result in a virtual preclusion of emotional reactions to complex situations, without impairment of primary emotions^{8,9}. It is tempting to suggest that whereas hyper- or hypoactivity produce abnormal moods, ablation of this region precludes them. Incidentally, my colleagues and I have observed that small irritative lesions in and about the target region cause episodes of anger and aggressive behaviour — a possible parallel to the link between PET activation and mania noted by Drevets *et al.* Third, anticipatory autonomic responses to possibly adverse response options are precluded by such lesions¹⁰. Finally, the ability to reason and decide advantageously is severely compromised by such lesions^{8,11,12}. Needless to say, depressive or manic states also compromise advantageous decision-making.

Taken together, these findings indicate that the human ventromedial prefrontal cor-

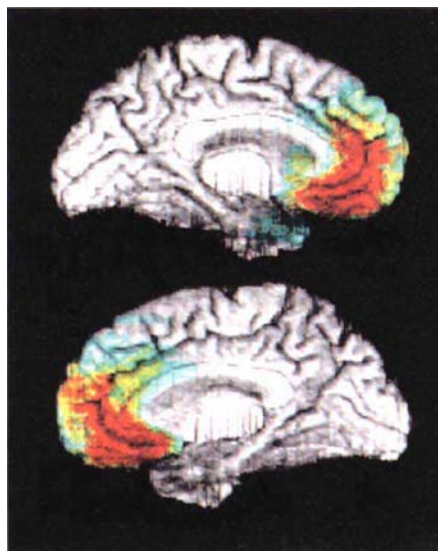


Figure 1 Drevets *et al.*¹ have identified a region of the prefrontal cortex that is abnormal in unipolar depressives (who suffer from depression only) and bipolar depressives (in whom depression and mania alternate). Bilateral damage in the sector to which this region belongs causes impairment of emotional responses to complex stimuli, and disturbances of decision-making, in neurological patients. The area in red corresponds to the maximal overlap of lesions in nine such patients and encompasses the region identified by Drevets *et al.* (Figure courtesy of Hanna Damasio, University of Iowa.)

tex is critical for the processing of emotions related to complex personal and social situations. Its dysfunction, be it caused by detectable lesions or by a still unspecified disorder of neural circuitry, alters the ability to reason and decide advantageously. Many exciting tasks lie ahead, aimed at specifying the relevant circuitry at the molecular and cellular levels, and at addressing intriguing system issues, such as the left/right asymmetry that is suggested by Drevets *et al.* and which has previously been hinted at in lesion studies of depression¹³. □

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1. Drevets, W. C. *et al.* *Nature* **386**, 824–827 (1997).
2. Van Hoesen, G. W., Morecraft, R. J. & Vogt, B. A. in *Neurobiology of the Cingulate Cortex and Limbic Thalamus* (eds Vogt, B. A. & Gabriel, M.) 249–284 (Birkhäuser, Boston, 1993).
3. Amaral, D. G. & Price, J. L. *J. Comp. Neurol.* **230**, 465–496 (1984).
4. Porrino, L. J., Crane, A. M. & Goldman-Rakic, P. S. *J. Comp. Neurol.* **198**, 121–136 (1981).
5. Van Hoesen, G. W. in *The Amygdaloid Complex* (ed. Ben-Ari, Y.) 77–90 (Elsevier/North-Holland, New York, 1981).
6. An, X., Bandler, R. & Price, J. L. *Soc. Neurosci.* **22**, 671 (1996).
7. Ongur, D., An, X. & Price, J. L. *Soc. Neurosci.* **22**, 672 (1996).
8. Damasio, A. R. *Descartes' Error: Emotion, Reason, and the Human Brain* (Grosset/Putnam, New York, 1994).
9. Damasio, A. R. *Neuroscientist* **1**, 19–25 (1995).
10. Bechara, A., Tranel, D., Damasio, H. & Damasio, A. R. *Cerebral Cortex* **6**, 215–225 (1996).
11. Damasio, A. R. *Proc. R. Soc. Lond. B* **351**, 1413–1420 (1996).
12. Bechara, A., Damasio, H., Tranel, D. & Damasio, A. R. *Science* **275**, 1293–1294 (1997).
13. Robinson, R. G., Kubos, K. L., Starr, L. B., Rao, K. & Price, T. R. *Brain* **107**, 81–93 (1984).

Daedalus

Thought versus feeling

Animals inhabit a sort of Garden of Eden. Everything they do, they do because they feel like it at the time. Their key motivation is excitement. Human beings have lost that holy simplicity. We have invented rational expectations, and with them, work — which is any activity, whether we feel like it or not, undertaken in the hope of useful results. Our most valuable motivation is interest.

However, excitement is making a powerful come-back. The electronic media try ever harder to saturate our lives with ersatz excitement, in the form of drama, sport, news, shoot-'em-up games, and so on. Children are urged to study science on the dishonest claim that 'science is fun'. Even *Nature* was recently redesigned to make it, including this column, more dramatic to look at but harder to read. The time has come, says Daedalus, to hit back against fun.

The victims of some poisonous snakes, such as cobras, are often seized with sudden detachment and lethargy, and give up without a fight. A toxin in cobra venom destroys the enkephalin responsible for arousal and excitement. Daedalus wants to isolate and synthesize this subtle venom compound, and test it as a drug. It, or one of its chemical relatives, should be a powerful antidote to fun.

DREADCO's 'Venium' will be the ideal counter to petty crime. Vandalism, rioting, bullying and graffiti-spraying are popular, not because they are profitable, but because they are fun. A depot injection of Venium would simply remove their appeal. Offenders should be given a dose adequate to destroy excitement while not initiating depression. With luck, the resulting mental vacuum would be filled with reflectiveness, curiosity and similar grown-up emotions. Other civilized enkephalins, hitherto outvoted by the primitive arousal-rewarders, might venture out of their vesicles. Safe from his animal promptings, the subject would be freed to explore his humanity.

Motorists, employees of bad bosses, insecure spouses and people plagued with evil temper will use Venium to defuse the terrible charm of confrontation. They will no longer snap at the lure of excitement and antagonism, and may be able to enjoy long trains of reflective thought. If Venium becomes popular enough, journalists and TV producers will cease the exhausting task of being endlessly exciting and dramatic, and will buckle down to the far more demanding and rewarding one of being interesting. And *Nature* could return to the drab format that built its greatness.

David Jones

A fungal minisatellite

Several minisatellites, or variable number of tandem repeats, have been described in the nucleotide sequences of mammalian¹, insect², fish³ and bird⁴ genomes. We present here sequence data of a minisatellite found in the lager brewing yeast *Saccharomyces carlsbergensis*. We were surprised to detect the minisatellite while investigating the degree of conservation between open reading frames (ORFs) in the yeasts *S. cerevisiae* and *S. carlsbergensis*⁵. This is, to our knowledge, the first description of a minisatellite nucleotide sequence from a fungus.

The minisatellite consists of 13 tandem repeats of 12 base pairs (Fig. 1), and is placed inside an ORF that is homologous to YCL010c from *S. cerevisiae*⁶. Alignment of the putative products from the two ORFs shows 70% identity at the amino-acid level. The *S. carlsbergensis* clone, 16BD1 (from C. Newlon and C. Yang) only supported the sequencing of supposedly the first 145 amino acids compared with 146 amino acids of YCL010c. Deletion of 12 of the 13 repeats in the *S. carlsbergensis* ORF allows alignment of the putative product to that of the *S. cerevisiae* ORF without gaps.

Alignment of the nucleotide sequence from the two ORFs shows two frame shifts. Deletion of one base pair, just downstream of the minisatellite, and insertion of one base pair further downstream, restores the frame. There is no stop codon created between them. The presence of the minisatellite within the *S. carlsbergensis* genome has been confirmed by cloning and partial sequencing of an independently obtained fragment.

The high degree of homology between the two ORFs indicates that they are derived from a common ancestor, and the fact that

only 12 of 13 repeats need be deleted to restore the alignment suggests that expansion of an existing DNA structure created the minisatellite. However, the possibility

5' -	1	GCCTTTCCCGAG	1
	13	GCCTTTCCCGAG	1
	25	GCCTTTCCCGAG	1
	37	GCCTTCCCTGGG	2
	49	GCCTTCCCTGGG	2
	61	GCCTTCCCTGGG	2
	73	GCCTTCCCTGGT	3
	85	GCTTTGCCTGGT	4
	97	GCTTTCCCTGGT	5
	109	GCTTTGCCTGGT	4
	121	GCCTTTCTGGT	6
	133	GCTTTGCCTGGT	4
	145	GCCTTTCCTGGC	7

Figure 1 DNA sequence of the Watson strand⁶ (polarity 5'→3') of the 156-base-pair minisatellite found in the brewing yeast, *Saccharomyces carlsbergensis*. The 13 repeats consist of 12 base pairs each and are aligned to give the highest homology between repeats. The numbers on the right represent the seven different repeat motifs found. The organization of the minisatellite shows a decreasing internal order going from top to bottom. Note that all repeats, except for those of type 1, contain the four-base-pair motif CCTG/CAGG (base pairs 8–11) which has been described in several minisatellites from various species^{1–4}. EMBL accession number is Z86109.

that the structure was created before the divergence of the sibling species cannot be ruled out, as a deletion covering 12 of the repeats might have occurred in the *S. cerevisiae* lineage.

The 156 base pairs that compose the minisatellite align over the entire length, with 71% identity and only 3 gaps, to the pl18 minisatellite⁴ from the bird *Phylloscopus trochilus*. Such a high degree of homology would normally indicate a close relationship between the two organisms. However, in this case it reflects that 8 of 12 base pairs of the consensus repeats in the two minisatellites are identical. Thus, a high degree of homology between minisatellite sequences does not necessarily represent a close phylogenetic relationship between two species. This emphasizes our general lack of knowledge about the origin of minisatellite DNA.

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1. Jeffreys, A. J., Wilson, V. & Thein, S. L. *Nature* **314**, 67–73 (1985).
2. Blanchetot, A. *Nucleic Acids Res.* **17**, 3313 (1989).
3. Bentzen, P. & Wright, J. M. *Genome* **36**, 271–277 (1993).
4. Gyllenstein, U. B., Jakobsen, S., Temrin, H. & Wilson, A. C. *Nucleic Acids Res.* **17**, 2203–2214 (1989).
5. Kielland-Brandt, M. C., Nilsson-Tillgren, T., Gjermansen, C., Holmberg, S. & Pedersen, M. B. In *The Yeasts* Vol. 6 (eds Wheals, A. E., Rose, A. H. & Harrison, J. S.) 223–254 (Academic, London, 1995).
6. Oliver, S. G. *et al. Nature* **357**, 38–46 (1992).

The one that got away

In his interesting News and Views article on our work¹, Paul Calvert compared the diffusion in thin polymer films to fish (unperturbed chains) swimming among reeds (fully stretched chains from either the air or substrate interfaces). We feel that this metaphor is misleading. What our results² and those of others³ show is that the diffusion coefficient is markedly reduced even when the thickness of the polymer film is more than 20 times the radius of gyration of the polymer. It is remarkable that surface effects could propagate so far into an amorphous polymer.

To form 'reeds', the chains must be anchored to the surface and the anchoring density would have to be quite high to overcome the entropic penalty of stretching the

chains away from their unperturbed gaussian state⁴. Achieving high grafting densities is quite difficult. In a melt, this entropic penalty is not offset by any favourable enthalpic interactions⁵.

Previous studies have shown that the gaussian conformation of the chain is retained even after grafting occurs⁶. Consequently, high grafting densities are unlikely. Even if a high grafting density could be achieved, the entropic penalty for a free chain to enter the grafted layer is simply too large. Thus, the 'fish' would be excluded from the reeds. Finally, if the reed picture were correct, thin polymer films would be optically anisotropic. This is not the case. Measurements from various laboratories suggest that, if anything, the chains have a preference for orienting parallel to the surface.

Each of these arguments dispels the

image of polymer fish swimming through a bed of polymer reeds. But the question still remains as to why the diffusion coefficient is suppressed at such large thicknesses. The phase diagram of polymer mixtures is also altered at comparable film thickness⁷. The Gibbs–DiMarzio theory of glasses treats the glass transition as being a second-order phase transition⁸. By analogy with the critical point for polymer mixtures, also a second-order transition, the glass transition in thin films may also be perturbed by finite size effects at similar distances. The diffusion coefficient is related to the difference between the glass transition and measurement temperatures. How much would the glass transition have to be changed to explain our results?

Based on bulk parameters for polystyrene, the diffusion coefficient would decrease by a factor of 2 at 140 °C by an

increase in the glass transition of only 7 °C. These arguments represent only one plausible explanation of the observed behaviour.

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1. Calvert, P. *Nature* **384**, 311–312 (1996).
2. Frank, B., Gast, A. P., Russell, T. P., Brown, H. R. & Hawker, C. *Macromolecules* **29**, 6531–6534 (1996).
3. Zheng, X. *et al. Phys. Rev. Lett.* **74**, 407–410 (1995).
4. Milner, S. T. *Science* **251**, 905–914 (1991).
5. Ligoure, C. & Leibler, L. *J. Phys. (France)* **51**, 1313–1328 (1996).
6. Zhao, X. *et al. Phys. Rev. Lett.* **69**, 776–779 (1992).
7. Reich, S. & Cohen, Y. *J. Polym. Sci. Polym. Phys.* **19**, 1255–1267 (1981).
8. Gibbs, J. H. & DiMarzio, E. A. *J. Chem. Phys.* **28**, 373–383 (1958).

Transcriptional activation functions in BRCA2

The *BRCA2* gene encodes a large protein of unknown function and is found mutated in 45% of familial breast cancers¹. Here we show that the portion of *BRCA2* encoded by its third exon shares homology with a known transcription factor and is capable of activating transcription, indicating a potential function of *BRCA2*.

In a search for any similarity to proteins with a known function, we found that exon-3 sequences at the amino terminus of *BRCA2* (within a region highly conserved between human and mouse) show sequence similarity to the activation domain of c-Jun (Fig. 1). The homology overlaps the δ -region of c-Jun which contains the binding site for the Jun N-terminal kinase (JNK)². We further investigated this similarity of exon 3 sequences to a transcriptional activation domain by assaying its transcriptional activation capacity. We found that *BRCA2* sequences spanning exon 3 (residues 23–105) have the potential to activate transcription in yeast, when linked to the *lexA* DNA-binding domain, whereas other highly conserved domains of *BRCA2* (residues 919–1,171; 1,500–1,589; 2,034–2,223; 3,200–3,326) do not show any activation potential in this assay (data not shown).

BRCA2 exon-3 sequences (residues 18–105) also have potent activation potential in two different mammalian cell lines, U2OS (Fig. 1) and NMuMG (data not shown) when these sequences are linked to the GAL4 DNA-binding domain. The c-Jun homology region (residues 60–105) contributes to this activation potential but has relatively little independent activity, whereas the adjacent region (residues 18–60) retains significant, although reduced, activation

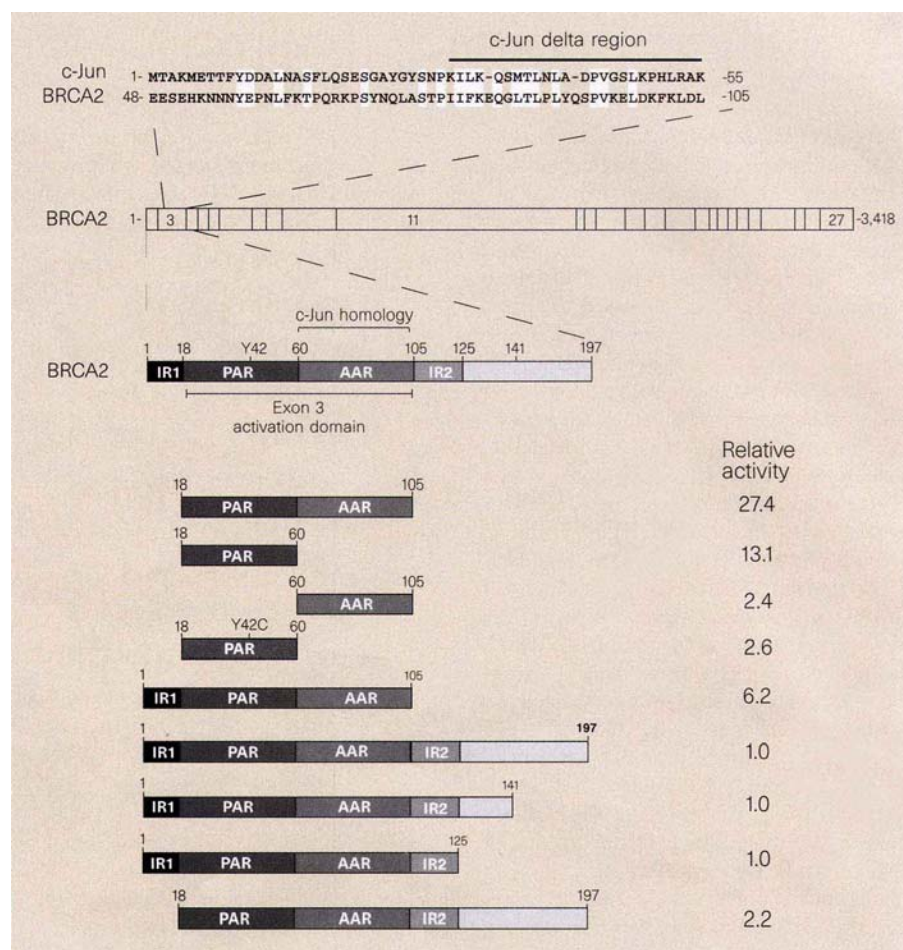


Figure 1 a, Sequences within exon 3 of *BRCA2* show similarity to the activation domain of c-Jun. Below the alignment is the exon structure of the *BRCA2* protein. PAR, primary activating region; AAR, auxiliary activating region; IR, inhibitory region. b, The various sequences of *BRCA2* were fused to the GAL4 DNA-binding domain (residues 1–147). These fusions were co-transfected into U2OS cells along with a target promoter 5GAL4EIBCAT, and CAT activity was measured 24 h after transfection. The activity shown is the relative value when compared with the activity of the GAL4 DNA-binding domain alone. The results are the average of at least three independent experiments. The expression levels of the different constructs were established by western blotting using a GAL4-specific antibody.

capacity. Residues 18–60 therefore represent a primary activating region whereas residues 60–105 represent an auxiliary activating region. At position 42 within the primary activating region there is a tyrosine residue which is mutated to cysteine in familial breast cancers³. Introduction of this Tyr-to-Cys mutation (Y42C) severely compromised the activation potential of the primary activating region.

Further characterization of this region reveals that the activation potential within exon 3 is under negative control of inhibitory regions (IR1 and IR2) present immediately on either side of exon 3. Together these small regions completely mask the activation potential of *BRCA2*. This type of regulation by inhibitor regions has been shown for a number of transcription factors^{4–6} and, in the case of c-Fos, inhibitor function was seen when the intact protein was assayed on its natural binding site⁴.

These results provide insight into the potential function and regulation of the

BRCA2 protein. They suggest that *BRCA2* has the ability to stimulate transcription, that its activation potential is under negative control by inhibitory sequences and that signal transduction pathways culminating in the stimulation of JNK-like kinases may regulate *BRCA2* activity.

To date, two genes have been implicated in the generation of familial breast cancers: *BRCA1* and *BRCA2*. Although the two protein products of these genes have no apparent sequence similarity, circumstantial evidence relating mainly to their size and overlapping expression patterns, has raised the possibility that their functions may be related⁷. Our finding that *BRCA2*, like *BRCA1*^{8,9}, has transcriptional activation potential provides the first functional evidence to support this hypothesis. Indeed, the fact that mutations found naturally in breast cancers disrupt the activation potential of both *BRCA1* and *BRCA2*, indicates that compromising this activity might be an important step in the generation of a subset

of familial breast cancers. Mutations found outside these activation domains may affect other, as yet unidentified, functions.

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1. Wooster, R. *et al. Science* **265**, 2088–2090 (1994).
2. Derijard, B. *et al. Cell* **76**, 1025–1037 (1994).
3. Friend, S. *et al. Nature Genet.* **11**, 238–239 (1995).
4. Brown, H. J., Sutherland, J. A., Cook, A., Bannister, A. J. & Kouzarides, T. *EMBO J.* **14**, 124–131 (1995).
5. Dennig, J., Beato, M. & Suske, G. *EMBO J.* **15**, 5659–5667 (1996).
6. Dubendorff, J. W., Whittaker, L. J., Eltman, J. T. & Lipsick, J. S. *Genes Dev.* **6**, 2524–2535 (1992).
7. Thakur, S. *et al. Mol. Cell. Biol.* **17**, 444–452 (1997).
8. Chapman, M. S. & Verma, I. M. *Nature* **382**, 678–679 (1996).
9. Varo, A., Monteiro, N. A., August, A. & Hanafusa, H. *Proc. Natl Acad. Sci. USA* **93**, 13595–13599 (1996).

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Infrared detection in a beetle

Buprestid beetles of the genus *Melanophila* approach forest fires in large numbers, from distances of up to 50 km, because their larvae can only develop in freshly burnt wood¹. Behavioural experiments have indicated that *Melanophila* do not use olfactory or auditory cues to detect forest fires². Using electrophysiological recordings we show here that the pit organs of *Melanophila* respond to infrared radiation, thus providing the first physiological evidence of an insect infrared receptor. Such a receptor could be used to identify the infrared emissions of a forest fire.

It is striking that only beetles of the genus *Melanophila* have paired thoracic pit organs. At the bottom of each pit are between 50 and 100 dome-shaped sensilla, which become fully exposed during flight (Fig. 1a). Each sensillum consists of a massive endocuticular sphere, which is suspended by a small apical stalk from the top of the outer cuticular dome (Fig. 1b, arrow) within a narrow cuticular cavity. The sphere is innervated by the ciliary dendrite of a single sensory cell, which has a tubular body at its tip (Fig. 1b, arrowhead). The ultrastructure of the sensory cell is therefore similar to that of a ciliary insect mechanoreceptor³.

Infrared irradiation of these sensilla causes non-flying *Melanophila* to twitch

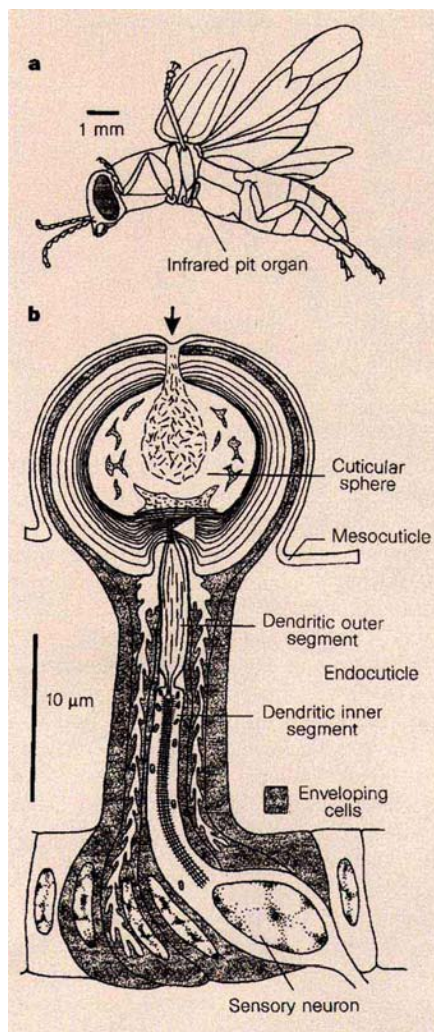


Figure 1 a, Diagram of *Melanophila* (body length 10 mm). The infrared pit organs, situated next to the coxae of the middle legs, are completely exposed during flight. b, An infrared sensillum, redrawn from ref. 3.

their antennae². In the wavelength range 2.5 μm to 4 μm — which corresponds to the emission maximum of a forest fire — an intensity of only 0.06 mW cm^{-2} is sufficient to cause twitching⁴. Although this behavioural response indicates that the pit organs of *Melanophila* might function as infrared sensors, there was, until now, no physiological evidence.

We recorded the activity of single units from individual sensilla of the pit organs of *Melanophila acuminata* by placing an electrolytically sharpened tungsten electrode close to a sensillum, a few micrometres into the cuticle. Impulses were amplified and recorded with standard apparatus. For stimulation we used the Oriel infrared element 6580 whose emission spectrum is similar to that of a forest fire. To rule out that heat was convected to the receptor, we focused an image of the infrared source onto the ventrolateral side of the thorax with a mirror. Between the mirror and the beetle we placed a camera shutter, a long-

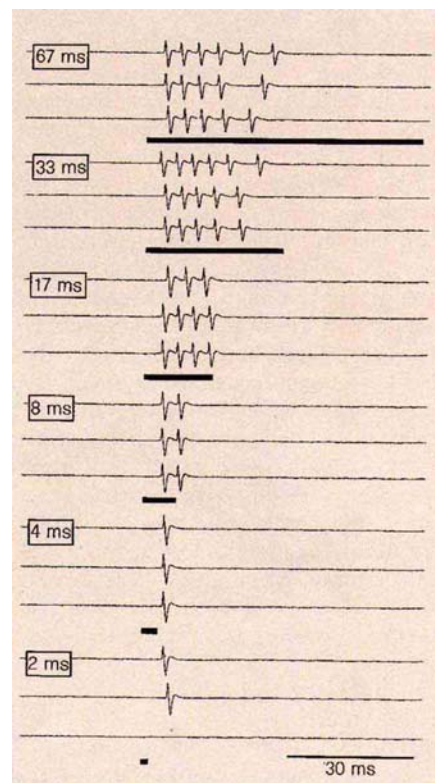


Figure 2 The responses of a neuron, recorded from the pit organ, to various infrared stimuli. Each trace shows the original response to one stimulus. Horizontal bars indicate exposure times. Each trial was repeated three times. The number of action potentials decreases with decreasing stimulus duration; 2 ms was sufficient to generate a response. If the mirror was covered, no response was recorded at any of the infrared intensities and shutter speeds tested.

pass infrared filter (50% cut-on at 1.8 μm) and neutral-density filters. At a radiation intensity of 24 mW cm^{-2} single neurons ($n = 40$) responded after a latency of 3–4 ms with up to six action potentials. A stimulus duration of 2 ms was sufficient to elicit one action potential (Fig. 2). Lower intensities (5 mW cm^{-2}) could elicit a neural response if the exposure time was longer (30 ms). If we interrupted the infrared beam with a chopper wheel, units responded with one phase-locked action potential per stimulus cycle, up to a frequency of 100 Hz.

Control experiments showed that the infrared receptors of *Melanophila* also respond to mechanical deformation, and could therefore function in a hitherto undocumented way. The spheres of the pit organ consist of organic molecules (endocuticle)^{2,3} which have C-H and O-H groups. Such groups have stretch resonances near 3 μm (ref. 5) and thus absorb strongly near this wavelength. Infrared absorption is converted into heat within fractions of a millisecond. This heating would cause a change in sphere volume, and we suggest that this might induce a deformation (compression or dilatation) of the dendritic tip.

A rough calculation of the heating and deformation of the sphere caused by infrared radiation of a few mW cm^{-2} for a few milliseconds gives an estimate for the membrane deformation in the order of 1 nm. A mechanical stimulus of this amplitude is sufficient to elicit a response from insect mechanoreceptors⁶. This proposed mechanism of transferring infrared radiation to mechanical action is used for photoacoustic spectroscopy⁷, an ultra-sensitive measuring technique in which an infrared beam thermally excites absorbing matter, in turn displacing the membrane of a loudspeaker.

Infrared detectors have previously been observed in crotalid and boid snakes, where infrared radiation warms up a thin membrane innervated by fibres of the trigeminal nerve. These act as true thermoreceptors⁸. In contrast, the stimulus for the sensory cell in the infrared receptors of *Melanophila* is a mechanical one, and the sensillum seems to function according to a photomechanical principle.

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1. Linsley, E. G. *J. Econ. Entomol.* **36**, 341–342 (1943).
2. Evans, W. G. *Nature* **202**, 211 (1964).
3. Vondran, T., Apel, K.-H. & Schmitz, H. *Tissue Cell* **27**, 645–658 (1995).
4. Evans, W. G. *Ecology* **47**, 1061–1065 (1966).
5. Herzberg, G. & Huber, K.-P. *Molecular Spectra and Molecular Structure. I. Spectra of Diatomic Molecules* (Van Nostrand and Reinhold, New York, 1950).
6. French, A. S. *Annu. Rev. Entomol.* **33**, 39–58 (1988).
7. Bicanic, D. D. *Photoacoustic and Photoacoustic Phenomena III* (Springer Series in Optical Sciences Vol. 69 (Springer, Berlin, 1992)).
8. Bullock, T. H. & Barrett, R. *Comm. Behav. Biol. A* **1**, 19–29 (1968).

Pet cat hair implicates murder suspect

DNA fingerprinting has been widely used as a forensic tool for over a decade^{1,2}, with simple tandem repeat (STR) loci³ being increasingly used to compare genotypes^{4,5}. Genomic and mitochondrial DNA, extracted from human or animal hairs collected at crime scenes, can be genotyped using the polymerase chain reaction (PCR)^{6–8}. By characterizing STR loci in domestic species, including cats and dogs^{9,10}, individual animals can be identified with high statistical confidence^{11,12}. As an example we describe a homicide case where the composite STR genotype of a hair linked to a crime scene matched that of the suspect's pet cat.

The case concerned a 32-year-old woman who disappeared from her home in Richmond, Prince Edward Island, Canada, on 3 October 1994. Her abandoned car was dis-

Table 1 Comparison of suspect (Snowball) and jacket hair (Evidence) genotypes

STR Locus	Allele Size (bp)		Allele size difference (bp)		STR match window* (bp)
	Snowball	Evidence			
FCA 026	147.83, 143.73	148.11, 143.73	0.28, 0.0	<	0.37
FCA 043	126.38, 120.52	126.29, 120.50	0.09, 0.02	<	0.59
FCA 080	259.27, 253.39	259.20, 253.14	0.07, 0.25	<	0.30
FCA 088A	121.91, 110.50	121.91, 110.50	0.0, 0.0	<	0.42
FCA 126	143.41, 141.08	143.41, 141.08	0.0, 0.0	<	0.53
FCA 132	152.73, 150.69	152.73, 150.69	0.0, 0.0	<	0.27
FCA 149	132.02, 128.05	131.80, 128.07	0.22, 0.02	<	0.29
FCA 058	229.03	229.24	0.21	<	0.36
FCA 090	93.54	93.54	0.0	<	0.46
FCA 096	210.95	211.00	0.05	<	0.25

*Match window gives the maximum allowable variation to declare a match, and was defined as the maximum pairwise allele size difference in a 70-member feline pedigree, comparing the migration at each locus of all alleles that are identical by descent¹⁴.

covered within days, and blood found inside was shown to match the victim. Three weeks later, a man's leather jacket stained with the victim's blood was discovered in woods, 8 km from her home. In the lining several white domestic cat hairs were found (A. E. Evers, Royal Canadian Mounted Police).

The victim's body was uncovered in a shallow grave on 6 May 1995, and the victim's estranged common-law husband was arrested and charged. The suspect lived with his parents and a pet cat; a white American shorthair named Snowball. We were asked to determine whether genomic DNA from the cat hairs found in the jacket matched Snowball's DNA profile.

Our laboratory had isolated and characterized nearly 400 dinucleotide STR loci from domestic cat DNA when constructing a linkage map of that species^{10,13}. We extracted and amplified DNA from the root of one of 27 hairs from the jacket and typed the DNA on the basis of ten feline dinucleotide repeat STR loci that were selected for optimal performance in forensic analysis¹⁴. We electrophoresed the fluorescently labelled PCR products in 6% denaturing polyacrylamide gels using a PE Applied Biosystems ABI 373A Automated DNA Sequencer. We similarly amplified DNA extracted from a blood specimen collected from Snowball (January 3 1995 by subpoena). Composite STR genotypes of the hair and Snowball's blood on the same gel were judged to match at all ten loci (seven heterozygous and three homozygous STR loci were resolved; Table 1).

The likelihood of a match between the hair genotype and a random individual was estimated from the frequency of the composite genotype in the population at large using allele frequency estimates from two STR population surveys: 19 unrelated cats from Prince Edward Island and nine cats from around the United States¹⁴. Although small, the island sample was adequate (95% confidence) to detect any STR allele present at a frequency of 9.5% or higher. The two populations showed appreciable allelic variation and remarkable population genetic similarity (as opposed to geographic population substructure). For example, 62% of the

detected STR alleles were present in both populations and the same alleles were most common for nine of the ten selected loci (all except FCA 088A) in both populations¹⁴.

The 10 STR loci were selected to reside on different linkage groups to ensure that there was no association between alleles at different loci. In addition, the distribution of outcomes of Fisher's Exact Tests on all pairwise combinations of 72 alleles between the 10 loci did not depart from expectations of independence in the island population sample (with Bonferroni correction for multiple tests). The incidence of the composite hair genotype for the seven heterozygous loci, estimated using the product rule and minimum allele frequency estimates for rare alleles^{11,12}, was 2.2×10^{-8} and 6.9×10^{-7} for the island and the US population databases, respectively.

The results of our analysis were presented and admitted to the Supreme Court of Prince Edward Island. The jury convicted the defendant of second-degree murder on 19 July 1996. The case represents a legal precedent for the introduction of automated STR genotyping of pet animal hairs in forensic cases that can be connected to the suspect in capital cases.

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1. National Research Council Technology in Forensic Science (Nat Acad., Washington DC, 1996).
2. Jeffreys, A. J., Wilson, V. & Thein, S. L. *Nature* **314**, 67–74 (1985).
3. Weber, J. L. & May, P. E. *Am. J. Hum. Genet.* **44**, 388–396 (1989).
4. Ziegler, J. S. *et al. Genomics* **14**, 1026–1031 (1992).
5. Kimpton, C. P. *et al. PCR Meth. Appl.* **3**, 13–22 (1993).
6. Higuchi, R., von Beroldingen, C. H., Sensabaugh, G. E. & Erlich, H. A. *Nature* **332**, 543–546 (1988).
7. Uchihli, R., Tamaki, K., Kojima, T., Yamamoto, T. & Katsumata, Y. *J. Forensic Sci.* **37**, 853–859 (1992).
8. Morin, P. A. *et al. Science* **265**, 1193–1201 (1994).
9. Ostrander, E. A., Map, F. A., Yee, M. & Rine, J. *Mam. Gen.* **6**, 192–195 (1995).
10. Menotti-Raymond, M. A. & O'Brien, S. J. *J. Hered.* **86**, 319–322 (1995).
11. Chakraborty, R. *Hum. Biol.* **64**, 141–159 (1992).
12. Budowle, B., Monson, K. L. & Chakraborty, R. *Int. J. Legal Med.* **108**, 173–176 (1996).
13. O'Brien, S. J., Wienberg, J. & Lyons, L. A. *Trends Genet.* (in the press).
14. Menotti-Raymond, M. *et al. J. Forensic Sci.* (in the press).

Ten ground squirrels dancing

The Kingdon Field Guide to African Mammals

by Jonathan Kingdon

Academic: 1997. Pp. 464. £29.95, \$39.95

Mark Pagel

At the launch of Jonathan Kingdon's book in Oxford's Natural History Museum, the description of an African wild dog was read from an old field guide: a "four-legged animal with a tapering snout". Modern 'wildlife tourists' may already know that much and demand more. Kingdon has responded by introducing an evolutionary biological emphasis to his guide. The result is a field guide concerned as much with understanding African mammals as with their identification.

The irony is that it has taken so long for this to happen, and if it even slightly nudges asidelist-ticking eagerness in favour of broad appreciation of the African ecosystem it will be a success. I am sure it will. Travellers and students of wildlife should take notice — this volume sets the standard for the field.

Two ways in which Kingdon brings the science of evolution to his book are by discussing 'adaptations' and 'genealogies' in his accounts of each species. He is the right author to have brought these ideas to field guides. A childhood in British East Africa and more than 25 years of producing atlases of the mammals have equipped Kingdon with a possibly unrivalled knowledge of his subjects, and this yields unusual and thought-provoking insights.

For example most see the "marbled"

markings of the African wild dog as obvious for camouflage. Kingdon rejects this, boldly suggesting that the "markings help to create a visual experience of inclusion and merging in the circling, clustering dogs during their 'meet' ceremonies". Kingdon avers that the unusually rapid growth rate of infant giraffes is an adaptation to escape predation. Users of the field guide may wish to attempt to fit this explanation alongside the equally unusually rapid growth of my three-year-old 'top-predator' son.

Genealogies describe the hierarchical branching pattern of evolution. Most readers will be familiar with the familial equivalent, the 'family tree'. One man in a southern shire of England has just traced his family tree back 9,000 years to a nearby cave. By putting genealogies into his field guide, Kingdon urges his readers to go beyond descriptions of how well an animal does in its environment. Genealogies wave at the reader a picture of who is related to whom and consequently of which features (adaptations) evolved from which others. They encourage the reader to grasp the great historical but anarchic march of evolution as it has produced new forms from old in response to changing environments and opportunities.

When one thinks of a field guide, it is pictures that first come to mind. In this context it is understandable why Kingdon's guide is called *The Kingdon Field Guide*. . . . As an artist and sculptor who works in abstract, impressionistic and illustrative styles, Kingdon brings an immediacy to his drawings that escapes all other field guides. The British art

critic Brian Sewell once said of the British National Gallery's thorough restoration of Holbein's *The Ambassadors* that "the future is secure". Sewell presumably meant that the possibility of sensitive restoration confers an immortality of sorts on a work of art. It's a pity that the same cannot be said of many African mammals. Kingdon at least secures the future of natural history illustration. The 1,150 or so mammals that earn a place in his book are not just represented by the usual police-style profiles, but also by pictures of gripping emotion and playful naughtiness — usually several, sometimes many, for each species.

Kingdon's reclining gorilla could be one's uncle on a Riviera beach, his alert Ader's duiker reveals the face of *The Hunted*, and his ten bush squirrels dancing on their hands conjure images of *The Twelve Days of Christmas*. Buy this book for yourself and for anyone else who likes nature just to know that you possess several thousand of Kingdon's wonderful drawings.

The 1849 resolution to create Oxford's Natural History Museum called for a building to house "all the materials explanatory of the organic beings placed upon the globe". By emphasizing evolutionary biology, *Kingdon's Field Guide* forsakes this Platonist legacy, and thereby stands alone in the genre. This is quite simply a superb and authoritative work by an author of unsurpassed credentials and talent for his task. Everybody will delight in it. □

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Kingdon 'secures the future of natural history illustration'. His dancing squirrels are of the *Paraxerus* and *Funisciurus* genera.

Does this man ever sleep?

Noam Chomsky: A Life of Dissent

by Robert F. Barsky

MIT Press: 1997. Pp. 237. \$27.50, £17.95

The world's most cited living person is a theoretical linguist. Noam Chomsky has published about 70 books and more than 1,000 articles, shaken up the study of natural language syntax several times, and won the Kyoto Prize for Basic Sciences, while supervising scores of PhD students at Massachusetts Institute of Technology (MIT), spending 20 hours a week on personal correspondence, lecturing all over the world, building a worldwide reputation as a radical critic of American foreign policy and media culture, and being father to three children. Does he ever sleep?

Robert Barsky's book does not tell us. It offers hardly any clear glimpses of Chomsky's personal life, despite being so rich in quotations from him that the blurb touts it as "the autobiography that Chomsky says he will never write". It has the faults of autobiography but not the virtues — there are scattered subjective judgements and unverified anecdotes from Chomsky without any connected first-person narrative to give a sense of him as a person.

Barsky does not compensate by providing competent intellectual biography. His letters from Chomsky are basically his only primary source (although a few photographs appear here for the first time). Barsky does not mention undertaking any interviews with people significant in Chomsky's life. He seems unacquainted not only with linguistics — references to Chomsky's work are in vapid phrases such as "conducting linguistic research that could lead us to a better understanding of the mind/brain" — but also with linguists. Two linguists' names (apart from Chomsky's) appear in his acknowledgments, and one of those is misspelled. Most of Chomsky's MIT colleagues — Bromberger, Flynn, Hale, Harris, Keyser, O'Neil — go unmentioned.

Barsky has simply not done the fact-checking and critical analysis that we expect from a biographer. The 1958 Texas conference at which Chomsky debated with leading establishment opponents is described by Chomsky in a letter written 37 years later; Barsky quotes no other participant. The section on "Chomsky as teacher" cites no discussions with any of his 60 or so doctoral students. Barsky just recycles three quotations from women graduates about whether Chomsky is a sexist, taken from a 1988 magazine article.

Interesting questions about Chomsky's career remain for a conscientious intellectual biographer to explore; for example, the

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Chomsky: author of more than 1,000 articles.

almost total absence of refereed journal papers from his voluminous publications (essentially all his work seems to be published by invitation), or his baffling insistence that he has been shunned and ignored throughout his career. (In truth he has been welcomed worldwide as a political speaker and has increasingly dominated formal linguistics — more than 80 per cent of the theoretical syntax papers at the last two conferences of the Linguistic Society of America were Chomskyan.) One will not find these topics examined in Barsky's book.

The writing is pretentiously sycophantic, from the opening sentence ("The task of writing a biography of Noam Chomsky gives new meaning to the word *daunting*") to the final verbless encomium ("Noam Chomsky, sixty-eight years old, institute professor, linguist, philosopher, grandfather, champion of ordinary people"). Between these bookends of cliché lie 215 pages of narrative ranging from soporifics such as "they were determined to provide a serene and comfortable life for their young children" to ludicrous verbiage such as "Humboldt and other enlightenment thinkers don't join the intellectual milieu surrounding and influencing Chomsky, they were always already there, waiting to be reilluminated".

Barsky does not even reilluminate the chronology of major events in Chomsky's life. The section called "The founding of MIT's graduate program in linguistics" forgets to tell us when the programme was founded (1959 or earlier is implied; actually the first regular graduate class entered in 1961). Events such as the 1975 Royaumont debate with Piaget or Chomsky's pivotal 1979 sabbatical in Pisa are overlooked completely.

Chomsky's political life dominates Barsky's perspective. Vastly more is said about Chomsky's links to radical leftist groups and Jewish political organizations than about his academic work. But, even in the political

arena, his life story is not adequately chronicled. The reader cannot find out from this book when it was (some time during the Vietnam War) that Chomsky visited Hanoi — or even that he went there at all. Nor can we find out from the section called "Chomsky and Montreal" whether he ever visited that city. Omitting the one fact that we might have expected, the section drifts off mysteriously into a discussion of Quebec fascism in 1942.

The most serious of Barsky's omissions is his failure to check Chomsky's recollections for accuracy or consistency. Chomsky's testimony about his celebrated academic war against "generative semantics" (GS), roughly from 1967 to 1975, amounts to denying that he even participated in it, which is an astonishing historical claim, even from a victor. Chomsky adds that "every single appointment" made in his department in the relevant period was of a GS practitioner. The idea that Chomsky, with his enormous prestige, could not have ensured the appointment of the colleagues he wanted cannot be taken seriously. He cites Paul Postal as an example of a GS hiring; but Postal left MIT in 1965, years before the battle over GS began.

Furthermore, Chomsky omits mention of Joan Bresnan — a major opponent of GS who was hired during 1974–75 with Chomsky's strong support. Then, in a later section, where a charge of running a patriarchal department is being discussed, he offers in defence his successful advocacy of Bresnan's appointment. Barsky does not explore the conflict between these passages (and fails to mention Bresnan in his index).

If Chomsky never writes an autobiography, and this amateurish cut-and-paste hack job is his biography, then the life of the most lionized intellectual of the twentieth century and most famous linguist in history will remain largely *terra incognita*. □

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Funding fathers

Der Stifterverband für die Deutsche Wissenschaft 1920–1995

by Winfried Schulze

Akademie Verlag: 1995. Pp. 388. DM78

The Stifterverband für die Deutsche Wissenschaft was founded in 1920 by representatives of German industry, banks and insurance companies to promote science in dire postwar times. Ever since, it has been an important source of funds for research in Germany. On 12 December 1995 the organization celebrated its seventy-fifth anniversary. To mark the occasion, Winfried

Schulze, a distinguished German historian from the University of Munich, was asked to write the "first integrated history" of the Stifterverband.

Schulze describes the position of the Stifterverband within the network of funding bodies that were created in Germany, such as the Kaiser-Wilhelm-Gesellschaft, the Helmholtz-Gesellschaft and the Notgemeinschaft der Deutschen Wissenschaft, the nucleus of what was to become the Deutsche Forschungsgemeinschaft (DFG). Special attention is paid to the question of how the Stifterverband fared during the period of National Socialism. The archives proved that the organization had a continuous life through these years, contrary to the memories of some witnesses. Unfortunately, we are not told what sort of activities were undertaken or what kinds of research projects were funded between 1933 and 1945.

But we are assured that "the Stifterverband persisted until 1945" — and was therefore justified in celebrating its seventy-fifth anniversary.

The postwar years were a time of complicated and controversial reorientation of the organization's politics. The agenda was dominated by decisions on what sort of scientific enterprises should be promoted, the relative shares that should be given to basic and applied science, and how funds should be allocated. Consensus persisted until the mid-1960s. The effects of a sharp economic recession bring the story up to date. Confronted with diminishing financial resources and a declining share of public research funding, the Stifterverband required a new strategy to make its activities more visible and to secure a stronger influence over the allocation of funds — a function that had been transferred to the DFG to a great extent.

The outcome of the debates was a programme based on two visions: subsidiarity and paradigmatic allocation of funds. The decision was made to promote the humanities, to strengthen interdisciplinary research and international cooperation and to work towards the reform of the German educational system. This framework has produced numerous important and innovative projects since the 1970s. It contributed to the shaping of the organizational, infrastructure and cognitive profile of parts of German science and humanities and laid the foundations of the reputation that the Stifterverband enjoys.

The author of a commemorative volume is confronted with a paradox that can be resolved only by a somewhat arbitrary decision. He or she may assume the perspective of the organization celebrating its jubilee and describe sympathetically its motives, goals and actions. Such an account will satisfy the curiosity of those who take an inter-

est in the 'life' of the subject. And it will remind those who are celebrating of some of their notable achievements — but these readers will not learn much about themselves from such an account. An alternative solution is for the observer to concentrate on the unintended consequences of strategies and actions. This is the perspective that social scientists prefer. But, again, it is not obvious what people or organizations can learn if they are confronted with the rationales and 'blind spots' of their actions and communications at the time.

Schulze chose the first alternative. So, although he gives a meticulous account of the Stifterverband's history, the reader is left with the impression that important questions have not been touched upon. I will concentrate on two of them. First, the historical study of public and private funding bodies is an important and welcome contribution to the empirical study of the evolution of science and humanities and the role played by the interactions between science, the economy and politics.

A reader expects to learn what experiences have been made with spending money on scientific investigations and how the

organizations have observed the effects and reacted to them. Little of that can be found in Schulze's book.

Second, since Schulze takes the perspective of the actors, his historical reconstruction results in recapitulation. That the Stifterverband is an institution striving for "the stimulation of science" could be learned from its annual reports and interviews given by Manfred Erhard, the general secretary of the association.

Because of the book's perspective, one cannot even put to an empirical test obvious hypotheses, such as that the special function of the Stifterverband today is not that of directing science by spending money.

An alternative explanation would be that the Stifterverband offers opportunities and events for the building of personal networks transcending social boundaries, so exerting an influence in the process of naming the problems and setting the agenda for the future organization of research and education. □

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Cultural cliffhanger

Spectacular cliff dwellings and ruined pueblos are among the remarkable monuments of America's past cultures that are to be found in the southwestern United States and northern Mexico. Archaeologist Stephen Plog has spent decades working in the region and has now written an account of the rise and mysterious

fall of these early cultures — the predecessors of the modern Hopi and Pueblo Indians. This picture shows White House Ruin, a thirteenth-century cliff dwelling in Canyon de Chelly National Monument in Arizona. *Ancient Peoples of the American Southwest* is published by Thames and Hudson (£18.95, \$27.50).

Soft cell

Life Itself

by Boyce Rensberger

Oxford University Press: 1997. Pp. 290. \$30, £21.95

Jeremy Hyams

The list of past students of the physiology course at Woods Hole Oceanographic Institution in Massachusetts reads like a who's who of cell biology. In the summer of 1987, the student body included the *Washington Post* science writer, Boyce Rensberger. The four weeks he spent in the beautiful surroundings of the Marine Biology Laboratory were to prove the start of two love affairs — one with a fellow student whom he subsequently married, the other with the living cell. *Life Itself* is the product of the second infatuation.

Cell biology is a field unusually rich in excellent textbooks. Most are written by cell biologists for cell biologists. They are mostly lavishly illustrated and written with a measured authority befitting the status of their often highly distinguished authors. *Life Itself*

is like none of these. Although its structure is fairly classical, with chapters on membranes, organelles, DNA structure, protein synthesis, mitosis and so on, the style certainly is not.

For Rensberger, a macrophage is a "great hulking predator brooding silently in the dim light waiting for some signal that prey had blundered into its vicinity". Platelets are the body's "911 Rescue Squad, a SWAT team, a Rapid Deployment Force, a Red Cross, and a few other emergency response mechanisms". The nucleus is a "giant brooding dome off to one side of the cell" (later it's a Volkswagen Beetle or the yolk in a fried egg). Chaperones work "like a sculptor modifying an unsatisfactory clay figure... massaging the amino acid chain, nudging a helix sideways, or shifting a loop from this side to that". The endoplasmic reticulum is a "hot air balloon". Microtubules grow out from centrioles, "making them look like two dandelions gone to seed".

The recurring theme is the cell as a living room, with the nucleus like the Beetle parked to one side (presumably there is an integral garage — or perhaps I am starting to take all this a bit too seriously). Membranes are the walls, and the organelles are sausages, grape-

fruit and basketballs. The sperm head is the size of an overstuffed chair and the sperm must swim nearly 2,000 miles, "drawn by the egg's perfume!". Perhaps the style owes more to *Alice in Wonderland* than it does to *Molecular Biology of the Cell* but, once one has convinced oneself that it is not some elaborate joke, it becomes quite compelling.

Rensberger's fascination with cells clearly also applies to the people who study them. He frequently resorts to the journalist's stock in trade, the snappy quotation. Many famous cell biologists make cameo appearances to offer personal comments on their particular specialities. Although some of the descriptions are rather lurid, what cannot be disputed is Rensberger's wonderment and enthusiasm because it literally leaps off the page. Here is the little boy who has been locked in the chocolate factory overnight, determined to gobble up as much as possible before the morning shift arrives and sends him home to his mum. It is all good, clean fun and, in truth, a rather remarkable achievement for a non-professional. (I hesitate to call someone who has managed to publish a picture of a cytoskeleton on the front page of a serious national newspaper an amateur.)

I am not entirely sure who *Life Itself* is aimed at. Presumably it is not intended to go up against the established cell biology tomes but it is hard to imagine a better way to convey to students the thrill of looking down a microscope at a living cell — and they get a pretty respectable introduction to cell structure and function thrown in for good measure. If Rensberger's other love affair is as successful as this one, he is a lucky chap. □

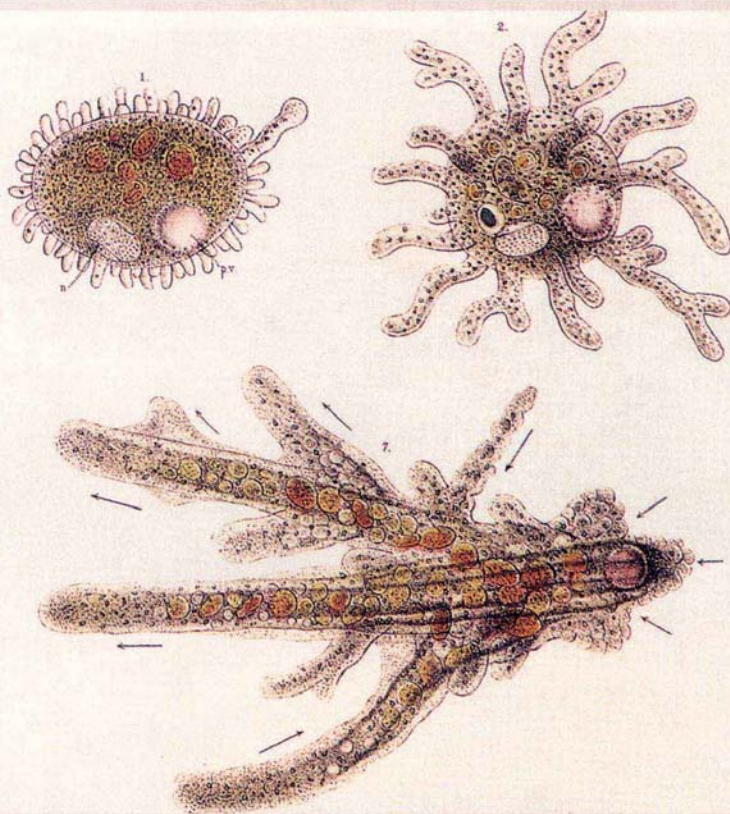
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New journals

This year, *Nature's* annual new journals review supplement will appear in the issue of 11 September. Publishers and learned societies are invited to submit journals for review, as well as details of any eligible electronic journals, taking note of the following criteria:

- Journals must have first appeared during or after June 1995 and issued at least four separate numbers by the end of May 1997.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year.
- Deadline for submission is 6 June.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates. For further information contact Peter Tallack, *Nature*, Macmillan Magazines, Porters South, Crinan Street, London N1 9XW, UK. Tel: +44 (0)171 843 4567. e-mail: p.tallack@nature.com.



The little changeling

The common freshwater amoeba was first described in 1755 by the German naturalist and miniature painter Roesel von Rosenhof who called it "der kleine Proteus" after the Greek sea god who could change his shape at will. It was given its official name *Amoeba proteus* by the American Joseph Leidy, whose beautifully illustrated *Fresh-water Rhizopods of North*

America, a plate of which is reproduced here, was published in 1879. The picture is one of 60 drawings and photographs chosen by Joseph G. Gall in *Views of the Cell: A Pictorial History* (American Society for Cell Biology, \$29). With the author's accompanying descriptions and historical analysis, the images pay homage to the pioneers of microscopy and cell biology.

Nucleocytoplasmic transport: signals, mechanisms and regulation

Erich A. Nigg

In eukaryotic organisms, DNA replication and RNA biogenesis occur in the cell nucleus, whereas protein synthesis occurs in the cytoplasm. Integration of these activities depends on selective transport of proteins and ribonucleoprotein particles between the two compartments. Transport across the nuclear envelope occurs through large multiprotein structures, termed nuclear pore complexes. It is signal-mediated and requires both energy and soluble factors, including shuttling carriers. Here I summarize current understanding of nucleocytoplasmic transport and illustrate the importance of regulated transport for signal transduction.

In eukaryotic cells, the nucleus is separated from the cytoplasm by a double membrane system known as the nuclear envelope. The resulting spatial segregation of major cellular activities requires efficient mechanisms for selective transport of proteins and nucleic acids^{1–5}, and it affords levels of regulation that do not exist in prokaryotes^{6,7}. In exponentially proliferating cells, hundreds of proteins and ribonucleoprotein particles (RNPs) are translocated through each nuclear pore complex (NPC) every minute. This extensive transport activity concerns proteins and several types of RNAs, hereafter collectively referred to as cargo (Table 1). The most prominent cargo for nuclear import consists of proteins, whereas export concerns mostly messenger RNAs, transfer RNAs, and ribosomal RNAs. Some RNAs, including many uridine-rich small nuclear RNAs (U snRNAs), are transported in both directions as part of their assembly into small nuclear RNP (snRNP) complexes⁴. Furthermore, many proteins shuttle continuously back and forth between the cytoplasm and the nucleus, some slowly^{8,9}, others rapidly^{10–12}. Nucleocytoplasmic transport of viral genomes is vital for the replication and assembly of many viruses¹³. Nuclear transport of human immunodeficiency virus (HIV), for instance, is critical for productive infection of non-dividing cells¹⁴.

Both proteins and nucleoprotein complexes are transported through NPCs, but the transport of different classes of cargo requires at least partly distinct factors. Several of these factors have now been identified, and both biochemical and genetic studies demonstrate that the basic mechanisms of nucleocytoplasmic transport have been highly conserved during evolution^{3,15,16}. These exciting new developments set the stage for studying the regulation of nucleocytoplasmic transport and exploring its relevance to cell physiology. My aim here is to review the emerging understanding of nuclear import and export, to identify major unresolved questions, and to illustrate the importance of nucleocytoplasmic transport for intracellular signal transduction.

The nuclear pore complex and nucleoporins

NPCs mediate bidirectional transport between the cytoplasm and the nucleus¹⁷. They provide aqueous channels of about 9 nm in diameter, which allow the diffusion of ions, metabolites and small proteins (relative molecular mass M_r less than $(40–60) \times 10^3$, 40K–60K), and mediate the selective transport of particles up to 26–28 nm in diameter by energy-dependent mechanisms^{3,18}. By electron microscopy, NPCs appear as roughly cylindrical structures, with eight-fold rotational symmetry in the plane of the nuclear envelope (Fig. 1)^{2,5,19,20}. At the available resolution of 60–100 Å, each NPC consists of a basic framework (a spoke complex embracing a central channel), positioned between a cytoplasmic ring and a nuclear ring

(Fig. 1a). The cytoplasmic ring is decorated by eight fibrils (Fig. 1a, b), and a basket-like assembly is attached to the nuclear ring (Fig. 1a, c). Vertebrate NPCs have an estimated outer diameter of 120 nm and M_r values of about 125,000K; they may thus contain approximately 1,000 proteins, with multiple (generally 8 or 16) copies of some 50–100 different proteins²¹. Many of these proteins, collectively termed nucleoporins, have now been characterized. Genes encoding vertebrate nucleoporins have been cloned primarily through the use of antibodies, and several nucleoporins have been localized to distinct NPC components by immuno-electron microscopy⁵. Most were found on either the cytoplasmic fibrils or the nuclear basket, and comparatively few on the basic framework (Fig. 1d–f). Yeast nucleoporins have been characterized by a combination of genetic and biochemical approaches^{18,22,23}. Extensive analyses of mutant phenotypes indicate a certain degree of functional overlap between individual nucleoporins but, interestingly, mutations in several essential nucleoporins inhibit nuclear import as well as export, consistent with the emerging view that these processes are coupled^{18,23}.

The primary structures of the known nucleoporins, as well as the phenotypes resulting from mutations of the corresponding genes, have been discussed in detail in several recent reviews^{5,21,23}. Here, only the most salient structural features of nucleoporins are summarized briefly. Many nucleoporins display highly repetitive motifs conforming to either the consensus FXFG and/or GLFG (amino acids are described using the single-letter code, with X indicating any amino acid). These FG-repeats interact *in vitro* with transport factors^{24–27}, but their precise functions *in vivo* remain to be clarified. It is attractive to speculate that different FG-repeats might differentially interact with distinct classes of cargo and thereby define separate transport pathways. Many nucleoporins also contain heptad repeats favouring α -helical coiled-coil formation, and these may promote the assembly of nucleoporin subcomplexes^{28–31}. Some nucleoporins (for example, mammalian nucleoporin (Nup)153 and Nup358) exhibit cysteine-rich zinc-binding motifs that are likely to mediate protein–protein or protein–nucleic acid interactions^{32–35}, whereas others (for example, yeast Nup100p, 116p and 145p) display octapeptide motifs also found in RNA-binding proteins³⁶. Whether these latter motifs reflect a function for the corresponding nucleoporins in RNA transport, or, alternatively, the presence of structural RNAs within the NPC, remains unknown. Similarly unclear is the significance of the attachment of O-linked N-acetylglucosamine to many vertebrate nucleoporins¹⁸.

Intriguingly, two nucleoporins (Nup98, Nup214) have been identified as fusion partners in distinct chromosomal translocations associated with myeloid leukaemia^{37,38}, and the N terminus of a

265K nucleoporin, termed Tpr (for translocated promoter region), has been found in oncogenic fusions with the kinase proto-oncogene products Met, Trk and Raf³⁹. This suggests that the fusion of certain nucleoporins to particular proteins might contribute to tumorigenesis. However, the molecular mechanisms by which this occurs remain to be clarified.

Signals for nuclear import and export

Localization signals have been identified for several classes of cargo, but by no means all (Table 1). Best understood are nuclear localization signals (NLS) responsible for targeting proteins to the nucleus. These were defined by systematic deletion and transfer experiments and in general are characterized by the presence of basic residues in either one or two clusters⁴⁰. Accordingly, these

NLSs are referred to as mono- or bipartite (Table 1). However, proteins may contain similar clusters of basic residues for reasons unrelated to nuclear transport, and, conversely, additional classes of (non-basic) NLSs may still await discovery⁴¹. Also, not every nuclear protein should necessarily be expected to possess its own NLS, as in some cases nuclear entry may be afforded by interactions with NLS-containing partners. On the other hand, histone proteins enter the nucleus by a NLS-mediated route, even though they would be small enough to traverse the NPC by diffusion⁴².

Considering that all proteins are made in the cytoplasm, yet many need to be imported into the nucleus, the existence of NLSs is easy to rationalize. It is perhaps less obvious why proteins should need nuclear export signals (NESs), as it would seem sufficient to prevent their import in the first place. Yet, efficient mechanisms for translocating proteins from the nucleus to the cytoplasm are expected to be important for the export of RNPs (see below), and in situations where the presence of a particular protein in the nucleus may be harmful to the cell. Notably in signal transduction, a protein's presence in the nucleus may be important at some times but deleterious at others. In line with these views, the first NESs were identified in HIV-1 Rev protein⁴³, and in a polypeptide inhibitor (PKI) of the cAMP-dependent protein kinase (PKA)⁴⁴. Rev is implicated in the export of viral RNA, whereas PKI most probably functions in the termination of signalling. The prototypic NESs of Rev and PKI are both short and hydrophobic, with a high leucine content (Table 1). Structurally related motifs are currently being identified in many distinct proteins. However, not every sequence motif functioning as a NES in a reporter construct should necessarily be expected to perform such a function in the context of the corresponding native protein.

The signals specifying transport of RNAs are not well defined, but are generally believed to reside with RNA-associated proteins. Binding of such proteins may depend on specific RNA secondary structures or modifications (Table 1). In the case of mRNA, export signals appear to be associated with abundant heterogeneous nuclear RNA-binding proteins (hnRNP proteins). In vertebrates, particular attention has been focused on A1, a hnRNP protein that shuttles rapidly between the nucleus and the cytoplasm¹⁰. Its transport in both directions depends on a 38 amino-acid motif, termed M9, indicating that import and export signals are either identical or interdigitated^{45–47}. When transferred to reporter proteins, M9 functions as both an NLS and an NES, yet its sequence bears no obvious similarity to either mono- or bipartite NLSs or to the NES of the Rev/PKI-type (Type 1). Export of 5S RNA in *Xenopus* oocytes depends on binding to either the ribosomal protein L5 or the transcription factor TFIIIA⁴⁸. Interestingly, TFIIIA displays a NES closely resembling those of Rev and PKI⁴⁹, but whether this NES is important for 5S RNA export is not known. In somatic cells, 5S RNA as well as rRNAs are exported in the form of preribosomal particles. Signals specifying export of rRNAs remain poorly understood, and the same is true for tRNAs^{4,13,50,51}.

A model emerges

Current models for transport through the NPC rest on three major premises: first, that distinct types of cargo contain specific molecular signals, as described above. Second, that signal-dependent translocation of cargo through the NPC is mediated by mobile (shuttling) carriers. And third, that most (albeit probably not all) transport processes require the small GTPase Ran. In essence, it is thought that soluble carriers bind cargo in one compartment, escort it through the NPC, release it in the other compartment, and shuttle back to the first compartment (Fig. 2). This general model is well supported for nuclear import; whether it also applies to export remains to be proved. In the case of protein import, carriers may be viewed as adaptors for mediating interactions between a large number of structurally distinct cargoes and a common, NPC-associated translocation machinery. In the case of RNP export, it

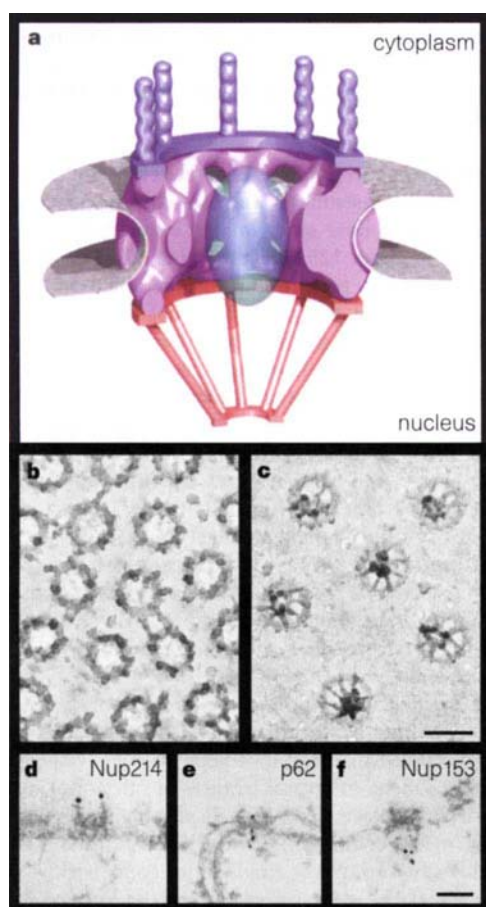


Figure 1 The nuclear pore complex (adapted from refs 5, 33, 136, 137). **a**, Schematic representation of the three-dimensional architecture of the NPC. The major structural components include the 52,000K basic framework (the spoke complex; adapted from ref. 19) in pink, the 12,000K central channel complex in translucent light blue, the 32,000K cytoplasmic ring with eight short, kinky fibrils in blue, and the 21,000K nuclear ring with attached nuclear basket in red. The cytoplasmic (**b**) and nuclear (**c**) periphery of the NPC are revealed by quick freezing/freezing rotary metal evaporation of spread *Xenopus* oocyte nuclear envelopes. **b**, On the cytoplasmic face of the NPC 'short cylinders' or 'collapsed filaments' are protruding from the massive cytoplasmic ring. **c**, On the nuclear face of the NPC eight thin filaments emanate from the more tenuous nuclear ring to join distally into a terminal ring, thereby forming a nuclear basket. **d–f**, Immunolocalization of Nups within the three-dimensional architecture of the NPC. Gallery of selected examples of colloidal gold labelled NPC cross-sections revealing the localization of distinct Nup epitopes: **d**, Nup214/CAN near the tips of the cytoplasmic fibrils; **e**, p62 at both the cytoplasmic and nuclear periphery of the central gated channel complex; and **f**, Nup153 close to or at the terminal ring of the nuclear basket. Scale bars, 100 nm (**b**, **c**) and (**d–f**).

is possible that similar adaptor functions might be provided by RNA-binding proteins escorting entire classes of RNAs. The shuttling carrier model is attractive for its simplicity, and it has the merit of focusing attention on discrete steps in the transport process. It is based on information obtained primarily from vertebrates and the budding yeast *Saccharomyces cerevisiae*. In the following, I will use a terminology applying to vertebrate homologues wherever possible, and, for the sake of clarity, each protein will be referred to by a single name, although many synonyms continue to be in use (for a guide to nomenclature, see Table 2).

A prominent role in nucleocytoplasmic transport has been attributed to the protein Ran, a member of the *ras* superfamily of small GTPases^{16,52,53}. Ran is highly abundant (10^7 copies per mammalian cell) and cycles between two forms, Ran-GTP and Ran-GDP (Fig. 3). The GTP- and GDP-forms of Ran bind selectively to distinct components of the transport machinery, and thereby control multiple protein-protein interactions important for cargo translocation¹⁶. Moreover, much of the energy requirement for nucleocytoplasmic transport can be attributed to GTP hydrolysis by Ran. This conclusion is based on the demonstration that a Ran mutant with a nucleotide specificity altered from GTP to XTP (xanthosine-5'-triphosphate) can support protein import in an *in vitro* system supplied with XTP even in the presence of non-hydrolysable analogues of ATP and GTP⁵⁴. However, an independent study using a similar approach reported evidence for an involvement of a second GTPase distinct from Ran⁵⁵. Thus, although protein import *in vitro* can apparently be driven by Ran only, it is possible that other GTPases may enhance transport efficiency or provide parallel import pathways. Also, the energy

requirements for nucleocytoplasmic transport *in vivo* remain unknown, and it would be premature to exclude rigorously a role for ATPases.

The low intrinsic GTPase activity of Ran is drastically stimulated by a GTPase-activating protein, termed RanGAP1. Conversely, the replacement of Ran-bound GDP by GTP is stimulated by a guanine-nucleotide exchange factor (GEF) termed RCC1^{3,16}. As illustrated in Fig. 3, these two regulators of Ran display a striking compartmentalization: the bulk of RanGAP1 is located in the cytoplasm, whereas RCC1 is predominantly nuclear. As a consequence, cytoplasmic Ran is expected to exist primarily in the GDP-bound state, whereas Ran-GTP is thought to be the prevailing form in the nucleus. However, the precise temporal and spatial distribution of the two forms of Ran is likely to be determined by selective interactions with several Ran-binding proteins (RanBPs)⁵⁶⁻⁶⁰. Both genetic and biochemical data demonstrate that Ran and its regulators are essential for nuclear import^{52,53,61-63}, and genetic studies suggest a role also in export⁶⁴⁻⁶⁷. However, as import and export processes are intimately linked (see below), *in vitro* studies will be required to prove an unequivocal role for Ran in export⁶⁸. Also, the functions of Ran and its regulators may not be limited to nucleocytoplasmic transport^{16,69}.

Mechanisms of nuclear import

A major breakthrough for the study of nuclear import was the development of efficient *in vitro* systems^{70,71}. One particularly powerful assay takes advantage of the fact that digitonin can be used to permeabilize cells while leaving the nuclear envelope intact⁷¹. *In vitro* import systems have allowed the characterization

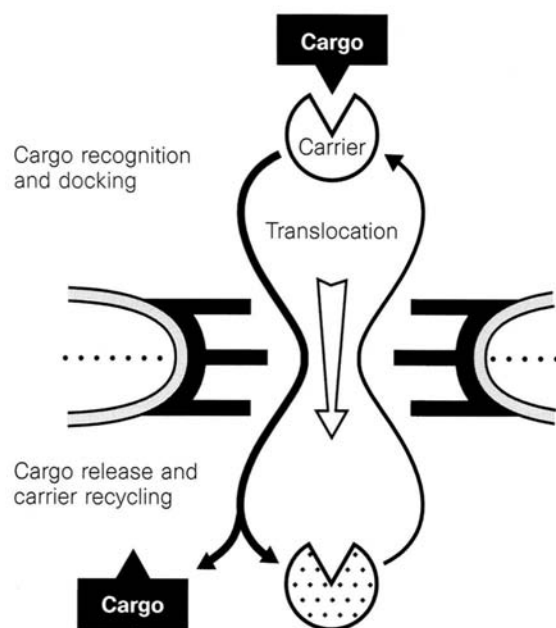


Figure 2 A unifying model for nucleocytoplasmic transport. This model emphasizes the role of shuttling carriers. It is well supported for protein import but largely speculative for RNP export. The NPC (in black) is shown schematically, embedded in the nuclear envelope (grey). Cytoplasmic fibrils and baskets are omitted and, as a consequence, cytoplasmic and nuclear sides are not defined.

Table 1 Cargo and signals for nuclear import and export

(a) Import cargo	
Proteins	Nuclear localization signals (NLS)*
SV40 T antigen	PKKKRKV (monopartite NLS)
nucleoplasmin	KRPAATKKAGQAKKKK (bipartite NLS)
c-myc†	PAAKR/KLD
RNAs	
U snRNAs	2,2,7-trimethyl guanosine (m ⁷ G) cap and Sm core proteins
5S RNA‡	NLS on ribosomal protein L5 (?)
(b) Export cargo	
Proteins	Nuclear export signals (NES)
PKI (Rat)	LALKLAGLDI
Rev (HIV-1)	LPPLERLTLD
TFIIIA (<i>Xenopus</i>)	SLVLDKLT
IκBα (Mouse)	QQLGQLTLENL
RNAs	
mRNAs	hnRNP proteins, such as hnRNP protein A1 (M9)
U snRNAs	7-methyl guanosine (m ⁷ G) cap, recognized by cap-binding proteins (CBC)
5S RNA‡	NES on TFIIIA or ribosomal protein L5 (?)
rRNAs (28S, 18S, 5.8S)	? (exported as preribosomal particles)
tRNAs	?
(c) Shuttling proteins	
Importin-α	
import signal (= importin β-binding domain)	
RMRFKFKNGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV	
export signal unknown	
hnRNP protein A1 (rapidly shuttling)	
import signal = export signal (M9)	
NQSSNFGPMKGGNFGGSSGPYGGGGQYFAKPRNQGGY	
nucleolin (slowly shuttling)	
import signal = bipartite NLS; no export signal	

This list provides examples and is not meant to be exhaustive.

* Amino-acid residues are indicated using the single-letter code.

† Bold letters mark residues that have been shown to be particularly important for signal function.

‡ Note that non-basic residues are critical for the function of the c-myc NLS.

§ In somatic cells, 5S RNA is exported as part of a preribosomal particle. In (*Xenopus*) oocytes, however, it undergoes a cycle of export-import, because of temporary storage in the cytoplasm.

Table 2 A nomenclature navigator

Names (vertebrates)	<i>Saccharomyces cerevisiae</i>	Function
(a) Components of the Ran-GTP/GDP cycle		
Ran (TC4)	Cnr1/Cnr2 (Gsp1/Gsp2)	Small GTPase
RCC1	Prp20p (Mtr1p, Srm1p)*	GEF for Ran
RanGAP1	Rna1p	GAP for Ran
RanBP1	Yrb1p	Stimulator of RanGAP1
RanBP2		NPC protein (NUP358)
(b) Shuttling carriers and transport factors		
Importin- α (karyopherin- α , PTAC58)	Srp1p/Kap60p/Rsl1pt	NLS-receptor
Importin- β (karyopherin- β , PTAC97)	Kap95p	Carrier (NLS proteins)
Transportin	YBR0224/Kap104p	Carrier (hnRNP A1)
NTF2 (p10, pp15, B-2)	Ntf2p	Cytoplasmic regulator of Ran function (?)
hRip1 (RAB1)	yRip1 (homologue?)	Binds to Rev NES; function unknown

Proteins involved in nucleocytoplasmic transport have been identified independently in different laboratories and multiple species, and synonyms have been coined in parallel. Only the most commonly used names are listed. Introduction of a unifying nomenclature would be helpful.

* Names for homologues of RCC1 and Ran in *Schizosaccharomyces pombe* are pim1 and spi1, respectively.

† Names for a *Drosophila* homologue of importin- α are pendulin/OHO31. Two distinct human importin- α subunits have been termed Srp1p (Np1p) and hSrp1 α 1 (Rch1p), respectively. For references see text and refs 3, 4, 16.

of several essential transport factors, the importance of which is corroborated by genetic studies in yeast^{3,15,16}. In brief, the docking of a NLS-protein to the NPC requires a heterodimeric carrier, whereas translocation through the NPC depends on the Ran-GTP/GDP cycle, and the small protein NTF2 (nuclear transport factor 2). This inventory is unlikely to be complete and additional transport factors probably await discovery. Permeabilized cells in fact still represent a complex system and factors will appear limiting only if they are efficiently removed during cell permeabilization. Figure 4 summarizes the major events involved in the nuclear import of a NLS-protein; these are discussed in some detail below.

Recognition of cargo by shuttling carriers and docking to the NPC. Proteins carrying either a mono- or a bipartite NLS are recognized by a soluble, heterodimeric carrier that consists of proteins of about 60 and 90K; in vertebrate species, these proteins have been given many names, including importin- α / β ^{72,73}, karyo-

pherin- α / β ⁷⁴, or pore targeting complex (PTAC) 58/97^{75,76} (for additional synonyms and names of yeast homologues see Table 2); hereafter they will be referred to as importin- α and importin- β . Both importins contain several hydrophobic 42 amino acid repeats (known as 'arm' repeats, named after the *Drosophila* gene *armadillo*). Importin- α binds to NLS-proteins, whereas importin- β strengthens the affinity of the complex for the NLS and mediates the docking of the cargo-carrier complex to the NPC^{25,73-75,77-79}. The cargo-carrier complex initially contacts the NPC at the distal end of cytoplasmic fibrils, from where it is transferred to the cytoplasmic entry of the central channel⁸⁰. Intriguingly, electron microscopy suggests that this transfer may involve bending (perhaps by brownian motion) of cytoplasmic fibrils⁸⁰.

Recent studies have revealed that the importin- α / β complex is not the only carrier involved in protein import^{81,82}. Specifically, nuclear import of the shuttling hnRNP protein A1 is mediated by an importin- β -related protein, termed transportin in mammals⁸¹ and Kap104p in yeast⁸². Transportin recognizes the M9 signal in protein A1, and presumably structurally related signals in other proteins, but does not interact with mono- or bipartite NLSs⁸¹. Most remarkably, transportin functions independently of an α subunit, although it is structurally related to importin- β . Thus, it is attractive to propose that importin- β might represent the archetype of a carrier protein, whereas importin- α might have evolved as an adaptor, favouring the recognition of a vast multitude of different NLS proteins.

Competition studies clearly demonstrate that transportin defines a second pathway for protein import⁸¹. In quantitative terms, the transportin and importin- α / β carriers may contribute to a comparable extent to the total flux of proteins to the nucleus. NLS proteins, as recognized by the importin- α / β carrier, may collectively be considered as a high-complexity class of cargo, with most members being present at comparatively low abundance. In contrast, mRNA-associated proteins are present in very large numbers (10^6 – 10^7 copies per cell), and thus represent a high abundance, low-complexity cargo. It is perhaps not surprising, therefore, that specialized carriers have evolved for the nucleocytoplasmic transport of such high-abundance cargoes. The genome of *S. cerevisiae* harbours only one gene for importin- α (*SRP1/KAP60*), but four distinct genes distantly related to importin- β . Of these, *Kap95* and *Kap104* are the likely homologues of mammalian importin- β and transportin, respectively^{77,82,83}. The other genes related to importin- β are expected to encode carriers for as yet unidentified cargoes. Metazoan organisms express not only multiple proteins related to importin- β , but also several importin- α isoforms³. Mammalian importin- α subunits show tissue-specific expression patterns and

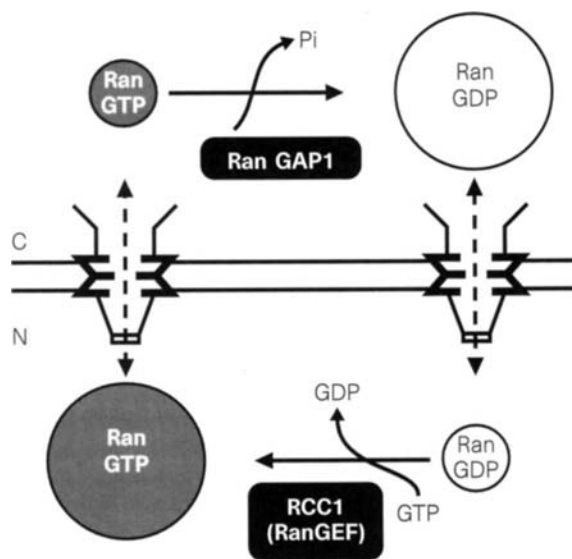


Figure 3 The Ran-GTP/GDP cycle. The model emphasizes the distinct nucleocytoplasmic localizations of major Ran regulators (RanGAP1 and RCC1), and the consequent expected asymmetry in the distributions of Ran-GTP and Ran-GDP. However, additional regulators of Ran may exist and the precise subcellular distributions of Ran-GTP and Ran-GDP have not been determined directly. NPCs are drawn with cytoplasmic fibrils and nuclear baskets.

may function as adaptors with at least partly distinct specificities for NLS proteins⁸⁴. In line with these views, mutational inactivation of one importin- α homologue of *Drosophila*, termed OHO31/pendulin, does not seem to impair protein import in all cells, but for some reason causes malignant transformation of haematopoietic cells^{85,86}.

Translocation through the NPC. Transfer of the cargo-carrier complex to the nucleoplasmic side of the NPC requires Ran-GDP on the cytoplasmic side, free GTP and NTF2. On the basis of *in vitro* protein-protein binding studies, it seems plausible that NTF2 may promote an interaction between cytoplasmic Ran-GDP, importin-cargo complexes and a subset of nucleoporins⁸⁷⁻⁸⁹, and that nucleotide exchange and GTP hydrolysis may occur on NPC-bound Ran^{54,58,61,90,91}. Another abundant, cytoplasmic protein implicated in linking the docking and translocation steps is Ran-binding protein 1 (RanBP1): this protein promotes the association of Ran with importin- β and enhances RanGAP1 activity^{57,60,66,92}, but its precise function remains unknown.

Translocation of the cargo-carrier complex through the NPC requires that a distance of about 100 nm be covered, and thus probably involves multiple steps. Several of these might require energy; alternatively, the actual energy-dependent translocation step could occur at a central structure of the NPC, and might require only a relatively small movement⁸⁰. *A priori*, it might have seemed attractive to invoke a participation of NPC-associated mechanochemical motor proteins, akin to myosin or kinesin. However, none of the known NPC genes codes for a recognizable motor, and most (if not all) energy consumption during protein import appears to reflect the GTPase activity of Ran, at least *in vitro*⁵⁴ (but see ref. 55). Thus, alternative models have been considered^{13,16,93}. In particular, it has been proposed that translocation might occur according to the 'brownian ratchet' hypothesis⁹⁴, which states that directionality may be conferred upon random thermal motions by chemical asymmetries. In the case of protein import through the NPC, one striking asymmetry stems from the distribution of Ran-GDP and Ran-GTP. Additional asymmetries may result from the disposition of individual nucleoporins. Furthermore, the affinity of importin- α for NLSs may be down-regulated selectively within the nucleus, and cargo may be recruited to non-diffusible sites, preventing its re-export. According to one specific model, translocation of NLS proteins might proceed by a series of docking and undocking reactions, involving multiple interactions between cargo-carrier complexes and FXFG-repeats in nucleoporins, and repeated rounds of Ran-GTP hydrolysis²⁴⁻²⁶. To assess the merits of the currently prevailing models, it will be important to determine to what extent cargo-carrier complexes dissociate during translocation, and how many molecules of GTP are hydrolysed per cargo translocated. At the heart of the problem, it

remains to be uncovered how the conceptual gating function of the NPC is reflected at the structural level. This may well require the development of new technologies, capable of monitoring motion with a high resolution in both time and space.

Dissociation of cargo-carrier complexes and carrier recycling. Once the cargo-carrier complex has reached the nucleoplasmic side of the NPC, the NLS protein is released, most probably through the action of nuclear Ran-GTP. In fact, the GTP form of Ran binds to importin- β , and thereby displaces importin- α as well as the NLS cargo^{25,92,95}. Moreover, an importin- β mutant which lacks the Ran-binding site is unable to discharge cargo into the nucleus⁶¹. Thus, the role of Ran is not limited to its GTPase activity. Instead, cytoplasmic Ran-GDP appears to be required at an early stage of NLS protein import, whereas nuclear Ran-GTP is implicated in terminating the translocation. In this fashion, the asymmetric distribution of Ran-GDP and Ran-GTP is likely to play an important role in determining the directionality of protein import. Following cargo-carrier complex dissociation, importin- β returns to the cytoplasm by unknown mechanisms, apparently without ever dissociating from the NPC^{61,73,96}. In contrast, both the NLS protein and importin- α are released into the nucleus. What mechanisms, other than dissociation from importin- β , promote the separation of importin- α from cargo is unknown, but phosphorylation might be involved⁹⁷. Also, the recycling of importin- α to the cytoplasm remains poorly understood, but clearly requires determinants distinct from those specifying nuclear import^{84,98}.

Nuclear import of other cargoes. In addition to proteins, nuclear import also concerns RNPs. Well studied examples include U snRNPs involved in splicing. Shortly after transcription, snRNAs U1, U2, U4 and U5 are in fact exported to the cytoplasm, where they acquire a set of specific proteins (known as Sm core proteins) and undergo additional methylations at the 5' cap, before they are imported back into the nucleus⁴. The binding of Sm core proteins is essential for nuclear import, whereas the formation of the trimethyl-guanosine cap (m₃G-cap) increases import efficiency, depending on the U snRNA or the cell type⁹⁹. How these two signals cooperate is not understood. Competition studies show that nuclear import of U snRNPs involves components that are partly distinct from those mediating NLS protein import, and these may include specialized, as yet unidentified carriers¹⁰⁰. Other requirements for transport, such as the Ran-GTP/GDP cycle, may be similar¹⁰¹.

Mechanisms of nuclear export

Progress towards understanding nuclear export has been hampered by the lack of efficient, well characterized *in vitro* systems. Furthermore, the study of RNA export is complicated by its tight coupling, both spatially and functionally, to pre-RNA processing^{3,4,13}. It is

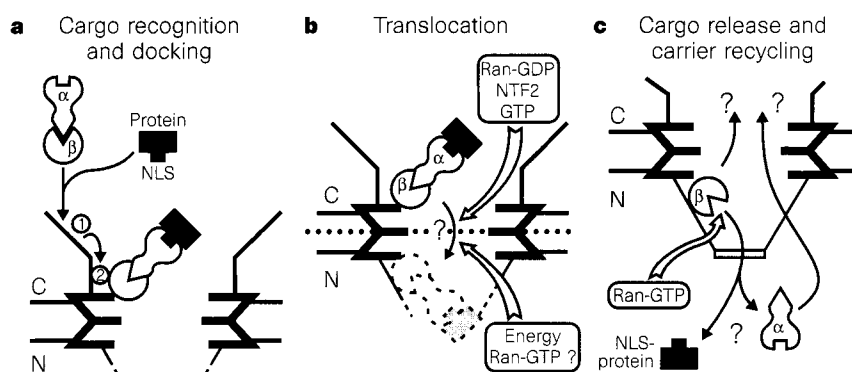


Figure 4 Protein import into the nucleus. **a**, NLS protein recognition by an importin- α / β heterodimer and docking to the NPC in a two-step reaction⁸⁰. **b**, Translocation of cargo-carrier complex; this step is poorly understood, but requires cytoplasmic Ran-GDP, NTF2, GTP hydrolysis by Ran, and perhaps

additional energy (at least *in vivo*). **c**, Termination of translocation by binding of Ran-GTP to importin- β , resulting in the release of importin- α and NLS protein into the nucleus. The mechanisms involved in the recycling of importin- α and - β to the cytoplasm are unknown. For further explanation see text.

difficult to isolate homogenous populations of fully processed nuclear RNPs, and the precise protein compositions of many RNP export substrates are unknown. Nascent pre-mRNAs, for instance, associate with about 20 abundant hnRNP proteins, of which several (including A1) accompany the mRNA to the cytoplasm, but others are specifically retained in the nucleus^{13,102}. Biochemical and genetic data suggest that hnRNP proteins play active roles in mRNA export^{46,103}. However, deciphering the precise roles of individual proteins will be difficult, because signals for RNA export may be additive, cooperative or partly redundant, and their importance may vary depending on the cargo. Also, multiple signals may be necessary for the translocation of large mRNPs.

In the absence of powerful *in vitro* systems, nuclear export has been studied by three complementary approaches. First, the large Balbiani ring transcripts in the salivary glands of the insect *Chironomus tentans* represent favourable objects for morphological studies on the processing and nuclear export of messenger RNPs^{104–106}. These studies have led to the important conclusion that hnRNPs unravel substantially while they transit through the NPC (Fig. 5). It is difficult to envision how such profound structural changes could be brought about without exertion of force within the NPC, although one could argue that RNPs might be pulled into the cytoplasm through the recruitment of their 5' caps onto ribosomes. Second, much information on the signals and factors involved in nuclear export has been obtained through microinjection of proteins or RNAs (or plasmids allowing transcription of RNAs) into the nucleus of *Xenopus* oocytes. This system has been used to demonstrate that RNA export is energy-dependent and saturable^{50,107}, and that export of distinct classes of RNAs (mRNAs, U snRNAs, tRNAs and rRNAs) involves at least partly class-specific factors, that is, perhaps distinct carriers^{51,108}. Third, valuable information about potentially relevant genes is emerging from the analysis of yeast mutant strains displaying defects in RNA export^{109,110}. At present, no detailed mechanistic models can be proposed for the export of any type of RNA but, as described below, significant progress has been made in a few specific areas.

Rev-mediated export of viral RNAs: hijacking of a cellular export pathway. Important insight into export mechanisms has come from studies on the viral Rev protein encoded by HIV-1^{43,111}, and its functional homologue Rex of human T-cell leukaemia viruses¹¹². In HIV-infected cells, Rev is essential for the cytoplasmic accumulation of incompletely spliced viral pre-mRNAs, and this function requires

Rev binding to a highly structured RNA motif termed RRE (Rev response element). Rev is a rapidly shuttling protein^{11,12} and it directs the nuclear export of RRE-containing RNAs, irrespective of whether or not these RNAs undergo splicing¹¹¹. Within its C-terminal domain, the viral Rev protein contains a NES⁴³ which structurally resembles the NESs identified in cellular proteins that are rapidly exported from the nucleus (Table 1). Introduction of the NES of Rev, coupled to bovine serum albumin, into *Xenopus* oocyte nuclei competitively inhibited nuclear export of 5S ribosomal RNA and U1 snRNA, but had no effect on the export of mRNA, tRNA or ribosomes⁴³. These and other data^{113,114} indicate that Rev functions as an adaptor for linking export of pre-viral RNA to a cellular export pathway, and that cellular cargo for this pathway may include 5S RNA–TFIIIA complexes, some U snRNPs, as well as several NES proteins (such as PKI, IκB) with no obvious role in RNA metabolism. So far, no carrier of the importin-β/transportin family has been implicated in exporting proteins with Rev/PKI-type NESs. However, cellular proteins able to interact with such NESs, and thus potentially involved in the corresponding export pathway, have been identified. These include human hRip/RAB1^{115,116} and yeast Rip1p¹¹⁷. Both proteins contain FG repeats reminiscent of nucleoporins, but display little sequence similarity otherwise. Yeast Rip1p appears to be concentrated at the nuclear envelope¹¹⁷, but the bulk of hRip/RAB1 is found in the nucleoplasm^{115,116}. Recent data suggest that NESs may interact with FG-repeats in several nucleoporins indicating that translocation of NES proteins through the NPC involves sequential interactions with particular nucleoporins²⁷.

Another protein with a Rev/PKI type NES has been identified in yeast¹¹⁸. This protein, termed Gle1p, was isolated in a screen for synthetic lethality with the GLFG-repeat NPC component Nup100. Mutational inactivation of the Gle1p NES impaired poly(A)⁺ RNA export, leading the authors to propose that Gle1p may be an essential mRNA export factor, and that Rev might mediate viral RNA export by mimicking Gle1p function¹¹⁸. However, this proposal appears to conflict with the finding that the NES of Rev did not compete with mRNA export in *Xenopus* oocytes⁴³, and further studies will be required to clarify the role of Gle1p.

A role for hnRNP proteins in mRNA export. Shuttling hnRNP proteins, exemplified by protein A1 of vertebrates^{46,106} and the structurally related Npl3p of yeast^{103,119}, have been proposed to play prominent roles in mRNA export¹³. In support of this view, the M9 signal confers export upon reporter proteins in the absence of

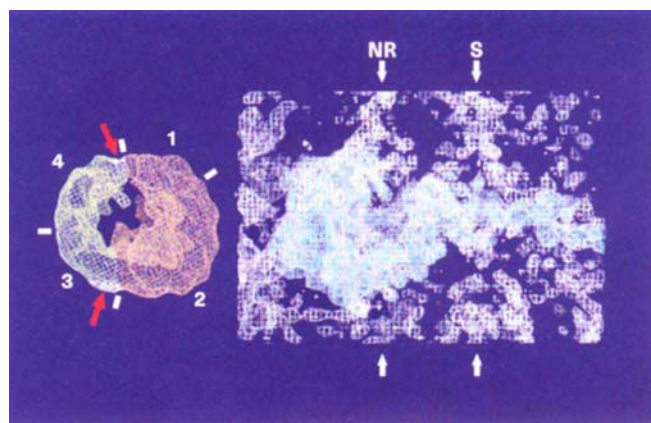


Figure 5 Transit of an RNP particle through the NPC. Three-dimensional reconstruction, based on electron micrographs, showing a Balbiani ring transcript RNP in transit through the NPC. The translocating particle (in light blue) is compared with a nucleoplasmic reference particle (yellow and brown). The surrounding material, including the NPC, is shown in white. The positions of the nuclear ring (marked NR) and the spokes (marked S) are indicated. The brown

part of the reference particle corresponds to the portion of the translocating RNP particle that has unravelled and entered the NPC, and the yellow part corresponds to the remaining globular portion. The contact regions between the translocating particle and the nuclear ring of the NPC are indicated on the reference particle as white patches (red arrows). Numbers indicate four domains of the RNP particle, as described previously¹⁰⁴. Adapted from ref. 104.

any RNA binding⁴⁶, and export of hnRNP protein A1 does not depend on ongoing RNA transcription, although, curiously, re-import does¹⁰². As described above, nuclear import of protein A1 is mediated by the carrier transportin⁸¹. Because the M9 signal specifies export as well as import⁴⁶, it will be interesting to explore the possibility that transportin might also mediate the export of RNP complexes carrying A1 proteins.

Cap-binding proteins in U snRNA export. The 5' ends of all RNA polymerase II transcripts, including pre-mRNAs and most U snRNAs, acquire a monomethylated guanosine (m⁷G) cap structure. This m⁷G cap is implicated in pre-mRNA processing and mRNA translation; in addition, it constitutes one of probably several signals important for export of U snRNAs^{108,120,121}. The m⁷G cap is recognized in the nucleus by a protein complex, termed CBC (cap-binding complex), which consists of two cap-binding proteins, CBP80 and CBP20¹²². So far, no export signals have been defined on CBPs, and the precise role of these proteins remains to be clarified. In particular, they are not required for viability in yeast. Furthermore, the m⁷G cap is not essential for export of mRNA, although CBPs do accompany hnRNPs through the NPC, perhaps contributing to ensure that the 5' cap takes the lead during hnRNP translocation¹⁰⁵.

Coupling of import and export

Import and export reactions are likely to be coupled at multiple levels. A first link stems from the fact that several proteins implicated in nucleocytoplasmic transport shuttle rapidly between the nucleus and the cytoplasm. Hence, sustained transport of cargo in one direction requires continuous recycling of carriers and factors in the other direction. A second link may be provided by Ran. Definitely required for protein import, the Ran-GTP/GDP cycle may be important also for the export of at least some classes of RNA¹⁶, albeit apparently not all^{69,123}. A recent provocative study suggests an unexpected, additional link between import and export¹²⁴. Specifically, both in yeast and in *Xenopus* oocytes, a nuclear complex between importin- α , CBC and m⁷G-capped RNA was detected, suggesting that CBC might shuttle while bound to importin- α . Most interestingly, cytoplasmic importin- β was shown to displace the importin- α -CBC complex from capped RNA, suggesting an attractive mechanism for releasing RNA cargo specifically in the cytoplasm¹²⁴.

Regulation of nucleocytoplasmic transport

Both nuclear transport rates and the functional sizes of NPCs are significantly greater in proliferating cells than in quiescent cells, and they are increased further in neoplastically transformed cells¹²⁵. This indicates that the NPC and/or the transport machinery are subject to controls that affect entire classes of cargo. These generalized controls are likely to relate to overall cell metabolism (for example, to events such as ribosome production); how they operate is not well understood. More specific types of regulation concern individual types of cargo. The best evidence for regulated RNA export stems from studies on virally infected cells. During adenovirus or influenza virus infection, for instance, export of viral RNA is favoured over export of cellular mRNA, and the cytoplasmic accumulation of early and late viral transcripts is also regulated at the level of export¹³. In the case of cellular RNAs, regulated export may contribute to determine not only the abundance, but also the spatial distribution of transcripts, particularly in response to stress (such as heat shock)¹²³ or during early development¹³. The mechanisms underlying these regulations are unknown. Export of proteins with NESs may often depend on protein-protein or protein-RNA interactions favouring the exposure of such signals⁴⁴. On the other hand, export of slowly shuttling proteins appears to be limited primarily by intranuclear retention⁹.

In the case of import, much attention has been focused on regulated nuclear entry of signalling proteins. For many prominent

signal transduction pathways, specific proteins have been identified that translocate from the cytoplasm to the nucleus in response to appropriate agonists (for example, hormones or growth factors), and often these proteins are kinases (or phosphatases) or transcription factors^{6,7,125}. It is beyond the scope of this review to survey signal transduction to the nucleus. Instead, the following brief discussion is meant to highlight a few general principles of regulated nuclear transport, and to illustrate its importance for cell physiology. At first glance, the nucleocytoplasmic distribution of signalling proteins appears to be controlled by many different mechanisms. Closer inspection shows, however, that most of these represent variations on two major themes: one is based on anchoring and release, the other on masking and unmasking of NLSs⁶. Both the release from anchoring sites and the functional activation of NLSs is brought about by similar mechanisms, particularly ligand binding, phosphorylation or proteolysis (or a combination of these).

One extreme example of anchoring is provided by transcription factors involved in cholesterol homeostasis. These factors, termed sterol-regulatory element binding proteins (SREBPs) are inserted into the endoplasmic reticulum membrane; thus, their liberation and nuclear translocation, in response to sterol depletion, requires proteolytic cleavage of the transmembrane domain¹²⁶. Membranes are involved also in the cytoplasmic anchoring of other transcription factors and of certain signalling protein kinases. The prototypic example for a protein kinase translocating to the cell nucleus is PKA⁶. Inactive PKA holoenzyme is anchored to cytoplasmic membraneous organelles. In response to appropriate hormonal stimulation, binding of cyclic AMP to regulatory subunits then releases active catalytic (C) subunits. These enter the nucleus, apparently by diffusion, where they phosphorylate CREB and other transcription factors. Binding of C subunits to PKI, the small polypeptide inhibitor of PKA described above, causes exposure of the NES of PKI and rapid export of the C subunit-PKI complex⁴⁴. Other protein kinases whose nucleocytoplasmic distribution changes in response to appropriate signals include MAP kinases and certain isoforms of protein kinase C; similarly, the cell-cycle regulatory protein kinase p34^{cdc2}/cyclin B undergoes a striking relocalization to the nucleus shortly before the onset of mitosis. However, the mechanisms controlling the redistributions of these latter kinases are not well understood.

Examples for masking and unmasking of NLSs are plenty, and in many cases NLS function is regulated by phosphorylation^{6,7}. Inactivation of a NLS may result from direct phosphorylation of residues within the NLS or in its immediate vicinity, as observed with the yeast transcription factor Swi5p whose nucleocytoplasmic distribution changes during the cell cycle¹²⁷. Alternatively, phosphorylation (or dephosphorylation) may cause conformational changes exposing a NLS, or may alter protein-protein interactions. Prominent examples for the latter mechanism are the NF κ B/Rel transcription factors¹³⁵. In this case, NLSs are masked by interactions with inhibitors of the I κ B family, and nuclear translocation of NF κ B/Rel requires inactivation of I κ B, usually by phosphorylation and proteolysis. Interestingly, I κ B appears to contain a NES, and may thus play a role also in terminating NF κ B signalling through a negative-feedback loop^{113,128}: once in the nucleus, NF κ B will in fact induce the synthesis of I κ B; newly synthesized inhibitor may then enter the nucleus, displace NF κ B from DNA and cause its export from the nucleus.

Finally, ligand-induced unmasking of NLSs has long been shown to control the nucleocytoplasmic distribution of nuclear receptors, such as those for glucocorticoid hormones. However, many nuclear receptors are likely to shuttle between the nucleus and the cytoplasm¹²⁹, and differences in steady-state distributions may reflect quantitative rather than qualitative differences in signalling mechanisms⁶. This may serve as a reminder that regulatory mechanisms based on the compartmentalization of signalling proteins should not be expected to control cellular responses in an all-or-nothing

manner. Instead, they are likely to modulate the kinetics and amplitudes of responses, and they are ideally suited for rapid signal transmission.

Conclusions and perspectives

Since the early 1980s, when the first evidence was obtained for signal-mediated nucleocytoplasmic transport^{107,130}, and NPCs were firmly established as gateways for translocation¹⁷, progress has been remarkable. The identification of shuttling proteins^{8,10} has fostered the realization that transport mechanisms may involve components that move back and forth between the nucleus and the cytoplasm. The establishment of permeabilized cell systems for studying protein import *in vitro*^{70,71} has allowed the functional characterization of soluble factors and shuttling carriers^{72,131,132}, and has brought to light the conspicuous role of the Ran-GTP/GDP cycle^{52,53}. In comparison, our understanding of RNA export remains rudimentary, and the development of reliable cell-free systems for studying export is eagerly awaited. Electron microscopy has shown that NPCs are beautiful structures^{5,19,20}, but the inner life of these structures remains a mystery, and detailed structural analyses of NPC components and transport factors by methods such as X-ray crystallography have barely begun^{133,134}. Also, answers to several fundamental questions are still lacking: for example, how many distinct transport pathways are there? Do they all use common factors, or do some operate on completely different principles? How many distinct carriers are there for protein import, and how many for export (if export uses carriers at all)? How is cargo translocated through the NPC, how is directionality achieved, and how are carriers recycled? Finally, how does cargo reach the NPC; does this occur by diffusion or does it involve structural tracks? Although these questions remain open, the newly acquired knowledge of transport mechanisms and their regulation already invites exploration of potential applications. In particular, it might be possible to target effector molecules to the nucleus, or conversely, block the nuclear translocation of particular signalling molecules, and thereby control the expression of specific genes, cell proliferation or differentiation. Furthermore, a better understanding of nucleoprotein transport during viral infection might prove useful for designing antiviral therapies and for designing delivery vectors for gene therapy. □

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- Gerace, L. Nuclear export signals and the fast track to the cytoplasm. *Cell* **82**, 341–344 (1995).
- Goldberg, M. W. & Allen, T. D. Structural and functional organization of the nuclear envelope. *Curr. Opin. Cell Biol.* **7**, 301–309 (1995).
- Görlich, D. & Mattaj, J. W. Nucleocytoplasmic transport. *Science* **271**, 1513–1518 (1996).
- Izaurrealde, E. & Mattaj, J. W. RNA export. *Cell* **81**, 153–159 (1995).
- Panté, N. & Aebi, U. Exploring nuclear pore complex structure and function in molecular detail. *J. Cell Sci. (suppl.)* **19**, 1–11 (1995).
- Nigg, E. A. Mechanisms of signal transduction to the cell nucleus. *Adv. Cancer Res.* **55**, 271–310 (1990).
- Karin, M. & Hunter, T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.* **5**, 747–757 (1995).
- Borer, R. A., Lehner, C. F., Eppenberger, H. M. & Nigg, E. A. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* **56**, 379–390 (1989).
- Schmidt-Zachmann, M. S., Dargemont, C., Kuhn, L. C. & Nigg, E. A. Nuclear export of proteins: the role of nuclear retention. *Cell* **74**, 493–504 (1993).
- Pinol-Roma, S. & Dreyfuss, G. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355**, 730–732 (1992).
- Meyer, B. E. & Malim, M. H. The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. *Genes Dev.* **8**, 1538–1547 (1994).
- Kalland, K. H., Szilvay, A. M., Brokstad, K. A., Saetrevik, W. & Haukenes, G. The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. *Mol. Cell Biol.* **14**, 7436–7444 (1994).
- Nakiely, S., Fischer, U., Michael, W. M. & Dreyfuss, G. RNA transport. *Annu. Rev. Neurosci.* **20**, 269–298 (1997).
- Bukrinsky, M. I. *et al.* A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* **365**, 666–669 (1995).
- Powers, M. A. & Forbes, D. J. Cytosolic factors in nuclear transport: what's importin? *Cell* **79**, 931–934 (1994).
- Koepp, D. M. & Silver, P. A. A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? *Cell* **87**, 1–4 (1996).
- Feldherr, C. M. & Akin, D. EM visualization of nucleocytoplasmic transport processes. *Electron Microsc. Rev.* **3**, 73–86 (1990).

- Davis, L. I. The nuclear pore complex. *Annu. Rev. Biochem.* **64**, 865–896 (1995).
- Hinshaw, J. E., Carragher, B. O. & Milligan, R. A. Architecture and design of the nuclear pore complex. *Cell* **69**, 1133–1141 (1992).
- Akey, C. W. & Radermacher, M. Architecture of the *Xenopus* nuclear pore complex revealed by three-dimensional cryo-electron microscopy. *J. Cell Biol.* **122**, 1–19 (1993).
- Bastos, R., Panté, N. & Burke, B. Nuclear pore complex proteins. *Int. Rev. Cytol.* **162B**, 257–302 (1995).
- Rout, M. P. & Blobel, G. Isolation of the yeast nuclear pore complex. *J. Cell Biol.* **123**, 771–783 (1993).
- Doye, V. & Hurt, E. C. Genetic approaches to nuclear pore structure and function. *Trends Genet.* **11**, 235–241 (1995).
- Radu, A., Moore, M. S. & Blobel, G. The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell* **81**, 215–222 (1995).
- Rexach, M. & Blobel, G. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell* **83**, 683–692 (1995).
- Iovine, M. K., Watkins, J. I. & Wente, S. R. The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. *J. Cell Biol.* **131**, 1699–1713 (1995).
- Stutz, F., Izaurrealde, E., Mattaj, J. W. & Rosbash, M. A role for nucleoporin FG repeat domains in export of human immunodeficiency virus type 1 Rev protein and RNA from the nucleus. *Mol. Cell Biol.* **16**, 7144–7150 (1996).
- Buss, F. & Stewart, M. Macromolecular interactions in the nucleoporin p62 complex of rat nuclear pores: binding of nucleoporin p54 to the rod domain of p62. *J. Cell Biol.* **128**, 251–261 (1995).
- Grandi, P. *et al.* A novel nuclear pore protein Nup82p which specifically binds to a fraction of Nsp1p. *J. Cell Biol.* **130**, 1263–1273 (1995).
- Grandi, P., Schlaich, N., Tekotte, H. & Hurt, E. C. Functional interaction of Nup96p with a core nucleoporin complex consisting of Nsp1p, Nup49p and a novel protein Nup57p. *EMBO J.* **14**, 76–87 (1995).
- Hu, T., Guan, T. & Gerace, L. Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. *J. Cell Biol.* **134**, 589–601 (1996).
- Sukegawa, J. & Blobel, G. A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell* **72**, 29–38 (1993).
- Panté, N., Bastos, R., McMorrow, I., Burke, B. & Aebi, U. Interactions and three-dimensional localization of a group of nuclear pore complex proteins. *J. Cell Biol.* **126**, 603–617 (1994).
- Wu, J., Matunis, M. J., Kraemer, D., Blobel, G. & Coutavas, E. Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J. Biol. Chem.* **270**, 14209–14213 (1995).
- Yokoyama, N. *et al.* A giant nucleopore protein that binds Ran/TC4. *Nature* **376**, 184–188 (1995).
- Fabre, E., Boelens, W. C., Wimmer, C., Mattaj, J. W. & Hurt, E. C. Nup145p is required for nuclear export of mRNA and binds homopolymeric RNA *in vitro* via a novel conserved motif. *Cell* **78**, 275–289 (1994).
- Kraemer, D., Wozniak, R. W., Blobel, G. & Radu, A. The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl Acad. Sci. USA* **91**, 1519–1523 (1994).
- Borrow, J. *et al.* The (7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. *Nature Genet.* **12**, 159–167 (1996).
- Byrd, D. A. *et al.* Tpr, a large coiled coil protein whose amino terminus is involved in activation of oncogenic kinases, is localized to the cytoplasmic surface of the nuclear pore complex. *J. Cell Biol.* **127**, 1515–1526 (1994).
- Dingwall, C. & Laskey, R. A. Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.* **16**, 478–481 (1991).
- Makkerh, J. P. S., Dingwall, C. & Laskey, R. A. Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. *Curr. Biol.* **6**, 1025–1027 (1996).
- Breuer, M. & Goldfarb, D. S. Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. *Cell* **60**, 999–1008 (1990).
- Fischer, U., Huber, J., Boelens, W. C., Mattaj, J. W. & Lührmann, R. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 475–483 (1995).
- Wen, W., Meinkoth, J. L., Tsien, R. Y. & Taylor, S. S. Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**, 463–473 (1995).
- Siomi, H. & Dreyfuss, G. A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.* **129**, 551–560 (1995).
- Michael, W. M., Choi, M. & Dreyfuss, G. A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell* **83**, 415–422 (1995).
- Weighardt, F., Biamonti, G. & Riva, S. Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. *J. Cell Sci.* **108**, 545–555 (1995).
- Guddat, U., Bakken, A. H. & Pieler, T. Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in *Xenopus* oocytes. *Cell* **60**, 619–628 (1990).
- Fridell, R. A. *et al.* Amphibian transcription factor IIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human immunodeficiency virus type 1 Rev. *Proc. Natl Acad. Sci. USA* **93**, 2936–2940 (1996).
- Bataillé, N., Helsner, T. & Fried, H. M. Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: a generalized, facilitated process. *J. Cell Biol.* **111**, 1571–1582 (1990).
- Pokrywka, N. J. & Goldfarb, D. S. Nuclear export pathways of tRNA and 40 S ribosomes include both common and specific intermediates. *J. Biol. Chem.* **270**, 3619–3624 (1995).
- Moore, M. S. & Blobel, G. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature* **365**, 661–663 (1993).
- Melchior, F., Paschal, B., Evans, J. & Gerace, L. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* **123**, 1649–1659 (1993).
- Weis, K., Dingwall, C. & Lamond, A. I. Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities. *EMBO J.* **15**, 7120–7128 (1996).
- Sweet, D. J. & Gerace, L. A GTPase distinct from Ran is involved in nuclear protein import. *J. Cell Biol.* **133**, 971–983 (1996).
- Coutavas, E., Ren, M., Oppenheim, J. D., D'Eustachio, P. & Rush, M. G. Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. *Nature* **365**, 585–587 (1993).
- Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W. & Ponstingl, H. Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO J.* **14**, 705–715 (1995).
- Melchior, F., Guan, T., Yokoyama, N., Nishimoto, T. & Gerace, L. GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.* **131**, 571–581 (1995).
- Dingwall, C., Daniels, Lewis, S. & Seraphin, B. A family of Ran binding proteins that includes nucleoporins. *Proc. Natl Acad. Sci. USA* **92**, 7525–7529 (1995).
- Chi, N. C., Adam, E. J. H., Visser, G. D. & Adam, S. A. RanBP1 stabilizes the interaction of Ran p97 in nuclear protein import. *J. Cell Biol.* **135**, 559–569 (1996).

61. Görlich, D., Panté, N., Kutay, U., Aebi, U. & Bischoff, F. R. Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.* **15**, 5584–5594 (1996).
62. Tachibana, T., Imamoto, N., Seino, H., Nishimoto, T. & Yoneda, Y. Loss of RCC1 leads to suppression of nuclear protein import in living cells. *J. Biol. Chem.* **269**, 24542–24545 (1994).
63. Corbett, A. H. et al. Ran/TC4 GTPase activating protein, is required for nuclear import. *J. Cell Biol.* **130**, 1017–1026 (1995).
64. Amberg, D. C., Fleischmann, M., Stagljar, L., Cole, C. N. & Aebi, M. Nuclear PRP20 protein is required for mRNA export. *EMBO J.* **12**, 233–241 (1993).
65. Kadowaki, T., Goldfarb, D., Spitz, L. M., Tartakoff, A. M. & Ohno, M. Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. *EMBO J.* **12**, 2929–2937 (1993).
66. Schlenstedt, G., Wong, D. H., Koeppe, D. M. & Silver, P. A. Mutants in a yeast Ran binding protein are defective in nuclear transport. *EMBO J.* **14**, 5367–5378 (1995).
67. Koeppe, D. M., Wong, D. H., Corbett, A. H. & Silver, P. A. Dynamic localization of the nuclear import receptor and its interactions with transport factors. *J. Cell Biol.* **133**, 1163–1176 (1996).
68. Morioanu, J. & Blobel, G. Protein export from the nucleus requires the GTPase Ran and GTP hydrolysis. *Proc. Natl Acad. Sci. USA* **92**, 4318–4322 (1995).
69. Cheng, Y., Dahlberg, J. E. & Lund, E. Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. *Science* **267**, 1807–1810 (1995).
70. Newmeyer, D. D., Finlay, D. R. & Forbes, D. J. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* **103**, 2091–2102 (1986).
71. Adam, S. A., Marr, R. S. & Gerace, L. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* **111**, 807–816 (1990).
72. Görlich, D., Prehn, S., Laskey, R. A. & Hartmann, E. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell* **79**, 767–778 (1994).
73. Görlich, D., Vogel, F., Mills, A. D., Hartmann, E. & Laskey, R. A. Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377**, 246–248 (1995).
74. Radu, A., Blobel, G. & Moore, M. S. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl Acad. Sci. USA* **92**, 1769–1773 (1995).
75. Imamoto, N. et al. In vivo evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. *EMBO J.* **14**, 3617–3626 (1995).
76. Imamoto, N., Tachibana, T., Matsubae, M. & Yoneda, Y. A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.* **270**, 8559–8565 (1995).
77. Görlich, D. et al. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* **5**, 383–392 (1995).
78. Weis, K., Mattaj, J. W. & Lamond, A. I. Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. *Science* **268**, 1049–1053 (1995).
79. Chi, N. C., Adam, E. J. & Adam, S. A. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* **130**, 265–274 (1995).
80. Panté, N. & Aebi, U. Sequential binding of import ligands to distinct nucleoporin regions during their nuclear import. *Science* **273**, 1729–1732 (1996).
81. Pollard, V. W. et al. A novel receptor-mediated nuclear protein import pathway. *Cell* **86**, 985–994 (1996).
82. Aitchison, J. D., Blobel, G. & Rout, M. P. Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science* **274**, 624–627 (1996).
83. Enekel, C., Blobel, G. & Rexach, M. Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J. Biol. Chem.* **270**, 16499–16502 (1995).
84. Weis, K., Ryder, U. & Lamond, A. I. The conserved amino-terminal domain of hSRP1 alpha is essential for nuclear protein import. *EMBO J.* **15**, 1818–1825 (1996).
85. Torok, I. et al. The overgrown hematopoietic organs-31 tumor suppressor gene of *Drosophila* encodes an importin-like protein accumulating in the nucleus at the onset of mitosis. *J. Cell Biol.* **129**, 1473–1489 (1995).
86. Küsel, P. & Frasch, M. Pendulin, a *Drosophila* protein with cell cycle-dependent nuclear localization, is required for normal cell proliferation. *J. Cell Biol.* **129**, 1491–1507 (1995).
87. Moore, M. S. & Blobel, G. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl Acad. Sci. USA* **91**, 10212–10216 (1994).
88. Paschal, B. M. & Gerace, L. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* **129**, 925–937 (1995).
89. Corbett, A. H. & Silver, P. A. The NTF2 gene encodes an essential, highly conserved protein that functions in nuclear transport in vivo. *J. Biol. Chem.* **271**, 18477–18484 (1996).
90. Paschal, B. M., Delphin, C. & Gerace, L. Nucleotide-specific interaction of Ran/TC4 with nuclear transport factors NTF2 and p97. *Proc. Natl Acad. Sci. USA* **93**, 7679–7683 (1996).
91. Nehrass, U. & Blobel, G. Role of the nuclear transport factor p10 in nuclear import. *Science* **272**, 120–122 (1996).
92. Lounsbury, K. M., Richards, S. A., Perlungher, R. R. & Macara, I. G. Ran binding domains promote the interaction of Ran with p97/beta-karyopherin, linking the docking and translocation steps of nuclear import. *J. Biol. Chem.* **271**, 2357–2360 (1996).
93. Panté, N. & Aebi, U. Toward the molecular dissection of protein import into nuclei. *Curr. Opin. Cell Biol.* **8**, 397–406 (1996).
94. Simon, S. M., Peskin, C. S. & Oster, G. F. What drives the translocation of proteins? *Proc. Natl Acad. Sci. USA* **89**, 3770–3774 (1992).
95. Morioanu, J., Blobel, G. & Radu, A. Nuclear protein import: Ran-GTP dissociates the karyopherin alphabeta heterodimer by displacing alpha from an overlapping binding site on beta. *Proc. Natl Acad. Sci. USA* **93**, 7059–7062 (1996).
96. Morioanu, J., Hijikata, M., Blobel, G. & Radu, A. Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl Acad. Sci. USA* **92**, 6532–6536 (1995).
97. Azuma, Y., Tabb, M. M., Vu, L. & Nomura, M. Isolation of a yeast protein kinase that is activated by the protein encoded by SRP1 (Srp1p) and phosphorylates Srp1p complexed with nuclear localization signal peptides. *Proc. Natl Acad. Sci. USA* **92**, 5159–5163 (1995).
98. Görlich, D., Henklein, P., Laskey, R. A. & Hartmann, E. A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. *EMBO J.* **15**, 1810–1817 (1996).
99. Marshall, C. & Lührmann, R. In vitro nuclear import of snRNPs: cytosolic factors mediate m3G-cap dependence of U1 and U2 snRNP transport. *EMBO J.* **13**, 222–231 (1994).
100. Michaud, N. & Goldfarb, D. S. Multiple pathways in nuclear transport: the import of U2 snRNP occurs by a novel kinetic pathway. *J. Cell Biol.* **112**, 215–223 (1991).
101. Palacios, I., Weis, K., Klebe, C., Mattaj, J. W. & Dingwall, C. Ran/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus. *J. Cell Biol.* **133**, 485–494 (1996).
102. Dreyfuss, G., Matunis, M. J., Pinol-Roma, S. & Burd, C. G. hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.* **62**, 289–321 (1993).
103. Lee, M. S., Henry, M. & Silver, P. A. A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. *Genes Dev.* **10**, 1233–1246 (1996).
104. Mehlin, H., Daneholt, B. & Skoglund, U. Structural interaction between the nuclear pore complex and a specific translocating RNP particle. *J. Cell Biol.* **129**, 1205–1216 (1995).
105. Visa, N., Izaurralde, E., Ferreira, J., Daneholt, B. & Mattaj, J. W. A nuclear cap-binding complex binds Balbiani ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export. *J. Cell Biol.* **133**, 5–14 (1996).
106. Visa, N. et al. A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell* **84**, 253–264 (1996).
107. Zasloff, M. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc. Natl Acad. Sci. USA* **80**, 6436–6440 (1983).
108. Jarmolowski, A., Boelens, W. C., Izaurralde, E. & Mattaj, J. W. Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* **124**, 627–635 (1994).
109. Amberg, D. C., Goldstein, A. L. & Cole, C. N. Isolation and characterization of RAT1: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.* **6**, 1173–1189 (1992).
110. Kadowaki, T. et al. Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (mtr) mutants. *J. Cell Biol.* **126**, 649–659 (1994).
111. Fischer, U. et al. Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J.* **13**, 4105–4112 (1994).
112. Bogerd, H. P., Fridell, R. A., Benson, R. E. & Cullen, B. R. Protein sequence requirements for function of the human T-cell leukemia virus type I Rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Mol. Cell Biol.* **16**, 4207–4214 (1996).
113. Fritz, C. C. & Green, M. R. HIV Rev uses a conserved cellular protein export pathway for the nucleocytoplasmic transport of viral RNAs. *Curr. Biol.* **6**, 848–854 (1996).
114. Fridell, R. A., Bogerd, H. P. & Cullen, B. R. Nuclear export of late HIV-1 mRNAs occurs via a cellular protein export pathway. *Proc. Natl Acad. Sci. USA* **93**, 4421–4424 (1996).
115. Fritz, C. C., Zapp, M. L. & Green, M. R. A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature* **376**, 530–533 (1995).
116. Bogerd, H. P., Fridell, R. A., Madore, S. & Cullen, B. R. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell* **82**, 485–494 (1995).
117. Stutz, F., Neville, M. & Rosbash, M. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell* **82**, 495–506 (1995).
118. Murphy, R. & Wente, S. R. An RNA-export mediator with an essential nuclear export signal. *Nature* **383**, 357–360 (1996).
119. Singleton, D. R., Chen, S., Hitomi, M., Kumagai, C. & Tartakoff, A. M. A yeast protein that bidirectionally affects nucleocytoplasmic transport. *J. Cell Sci.* **108**, 265–272 (1995).
120. Izaurralde, E., Stepinski, J., Darzynkiewicz, E. & Mattaj, J. W. A cap binding protein that may mediate nuclear export of RNA polymerase II-transcribed RNAs. *J. Cell Biol.* **118**, 1287–1295 (1992).
121. Terns, M. P., Dahlberg, J. E. & Lund, E. Multiple cis-acting signals for export of pre-U1 snRNA from the nucleus. *Genes Dev.* **7**, 1898–1908 (1993).
122. Izaurralde, E. et al. A cap-binding protein complex mediating U snRNA export. *Nature* **376**, 709–712 (1995).
123. Saavedra, C., Tung, K. S., Amberg, D. C., Hopper, A. K. & Cole, C. N. Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae*. *Genes Dev.* **10**, 1608–1620 (1996).
124. Görlich, D. et al. Importin provides a link between nuclear protein import and U snRNA export. *Cell* **87**, 21–32 (1996).
125. Feldherr, C. M. & Akin, D. Role of nuclear trafficking in regulating cellular activity. *Int. Rev. Cytol.* **151**, 183–228 (1994).
126. Wang, X., Sato, R., Brown, M. S., Hua, X. & Goldstein, J. L. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell* **77**, 53–62 (1994).
127. Moll, T., Tebb, G., Surana, U., Robitsch, H. & Nasmyth, K. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SW15. *Cell* **66**, 743–758 (1991).
128. Arenzana-Seisdedos, F. et al. Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. *Mol. Cell Biol.* **15**, 2689–2696 (1995).
129. Guiochon-Mantel, A. et al. Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J.* **10**, 3851–3859 (1991).
130. Dingwall, C., Sharnick, S. V. & Laskey, R. A. A polypeptide domain that specifies migration of nucleoplasm into the nucleus. *Cell* **30**, 449–458 (1982).
131. Adam, S. A. & Gerace, L. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* **66**, 837–847 (1991).
132. Moor, M. S. & Blobel, G. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell* **69**, 939–950 (1992).
133. Scheffzek, K., Klebe, C., Fritz Wolf, K., Kabsch, W. & Wittinghofer, A. Crystal structure of the nuclear Ras-related protein Ran in its GDP-bound form. *Nature* **374**, 378–381 (1995).
134. Bullock, T. L., Clarkson, W. D., Kent, H. M. & Stewart, M. The 1.6 angstroms resolution crystal structure of nuclear transport factor 2 (NTF2). *J. Mol. Biol.* **260**, 422–431 (1996).
135. Baeuerle, P. A. & Henkel, T. Function and activation of NF-kB in the immune system. *Annu. Rev. Immunol.* **12**, 540–566 (1994).
136. Jarnik, M. & Aebi, U. Toward a more complete 3-D structure of the nuclear pore complex. *J. Struct. Biol.* **107**, 291–308 (1991).
137. Panté, N. & Aebi, U. Toward the molecular details of the nuclear pore complex. *J. Struct. Biol.* **113**, 179–189 (1994).

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Effect of orogeny, plate motion and land-sea distribution on Eurasian climate change over the past 30 million years

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The Eurasian climates of today, 10 million and 30 million years ago are simulated using an atmospheric general circulation model that incorporates realistic continental geography and epicontinental sea distributions. The resulting climates compare well with various palaeoclimate records. The retreat of the Paratethys—an epicontinental sea—shifts the central Asian climate from temperate to continental conditions, and plays as important a role as uplift of the Himalayan/Tibetan plateau in driving the Asian monsoon changes.

Many atmospheric general circulation model (AGCM) simulations have shown the influence of topography on global climate through changes in atmospheric circulation^{1–7}. AGCM simulations ('experiments') have also investigated the effect of plateau uplift on global cooling during late Cenozoic times^{8–10}. The simulations were, in fact, sensitivity experiments involving the plateau height^{2,3,6,9–11} using a 'mountain/no-mountain' approach^{1,8}. They were, nevertheless, able to reproduce some trends of the regional and global changes, such as the enhancement of Indian and southeastern Asian monsoons¹², or the cooling of the Northern Hemisphere^{3,9,11}. New AGCM experiments, using more sophisticated land-surface hydrology schemes and a slab ocean, improved the comparison of model results and proxy data^{10,11}. However, none of these experiments have taken into account the temporal evolution of sea-land distribution resulting from plate tectonics.

Unlike previous studies^{6,9–11}, which mainly focused on plateau uplift, we have performed three AGCM experiments for present, Middle-to-Late Miocene (10 Myr ago) and Early Oligocene (30 Myr ago) climates (respectively Exp-1, -2 and -3) with a set of new palaeogeographical maps that include topography, a precise reconstruction of continents or blocks using palaeomagnetic data, and oceanic kinematic reconstructions. Changes in epicontinental sea coastlines are considered, including those of the Paratethys, a sea stretching over Eurasia 30 Myr ago which progressively receded during the Miocene period. To quantify separately the effect of the Paratethys shrinkage and the Tibetan plateau uplift, we have performed two sensitivity experiments: Exp-4 keeps an Early Oligocene palaeogeography without the Paratethys sea, while Exp-5 corresponds to the Middle-to-Late Miocene palaeogeography except for the Tibetan plateau height which is reduced to 900 m (the Himalayas remain unchanged). Comparing Exp-3 and Exp-4 will emphasize the effect of the Paratethys; a comparison of Exp-2 and Exp-5 will emphasize the effect of the plateau uplift. A third sensitivity test has been performed to investigate the effect of changes in sea surface temperatures.

Here we show that the Paratethys retreat plays a major role, causing the central Asian climate to change from oceanic to continental conditions. As the Paratethys shrinks, the amplitude of the seasonal cycle increases sharply and thus dramatically enhances monsoon precipitation. Another improvement in our

simulations is the use of different uplift rates for the Himalayas and the Tibetan plateau. This more realistic approach enables us to show that most of the increase in monsoon precipitation is located over the southern flank of the Himalayas whereas the western part of the Tibetan plateau becomes drier. Moreover, sensitivity experiments show that this geographical pattern of precipitation increase is mostly due to the Paratethys shrinkage. Prescribing accurate palaeogeography enables the model to be more sensitive to realistic forcing factors than were previous experiments^{6,9–11}, and also improves the agreement between model results and palaeoclimate indicators. In a second step, we coupled the AGCM with a mixed-layer ocean at lower resolution. We show that, although the amplitude of the modelled climate changes is sensitive to the surface ocean response¹⁰, the major climate changes that were simulated with prescribed SST remain unchanged.

Model simulations

Numerical experiments were performed using the Laboratoire de Météorologie Dynamique Version 5.3 AGCM¹³ which represents realistically present-day Asian monsoon⁵⁰. We have used the standard resolution, corresponding to 64 points regularly spaced in longitude, 50 points regularly spaced in the sine of latitude, and 11 vertical levels of normalized pressure coordinates. The model solves the dynamic and thermodynamic equations, and the continuity equations for mass and water vapour. Each experiment includes the full seasonal cycle and lasts 16 model years; averages are made over the last 15 years.

The major improvement over previous experiments is the use of more sophisticated palaeogeographical maps (Fig. 1a, b). Continents have been located with respect to the Earth's spin axis using oceanic finite reconstruction parameters^{14–16} and the palaeomagnetic pole positions of Besse and Courtillot^{17,18} (the hotspot reference frame is not used in this study). The shortening of the Asian lithosphere has also been simulated, using palaeomagnetic data to restore the southern margin of Asia^{19,20} progressively deformed by the penetration of India. Indochina was reconstructed with respect to China using the South China Sea kinematic parameters²¹. Palaeoshorelines are those drawn in the Tethys project palaeoenvironmental maps²². The most outstanding feature is the large areal extent of the Paratethys sea during the Oligocene and its gradual

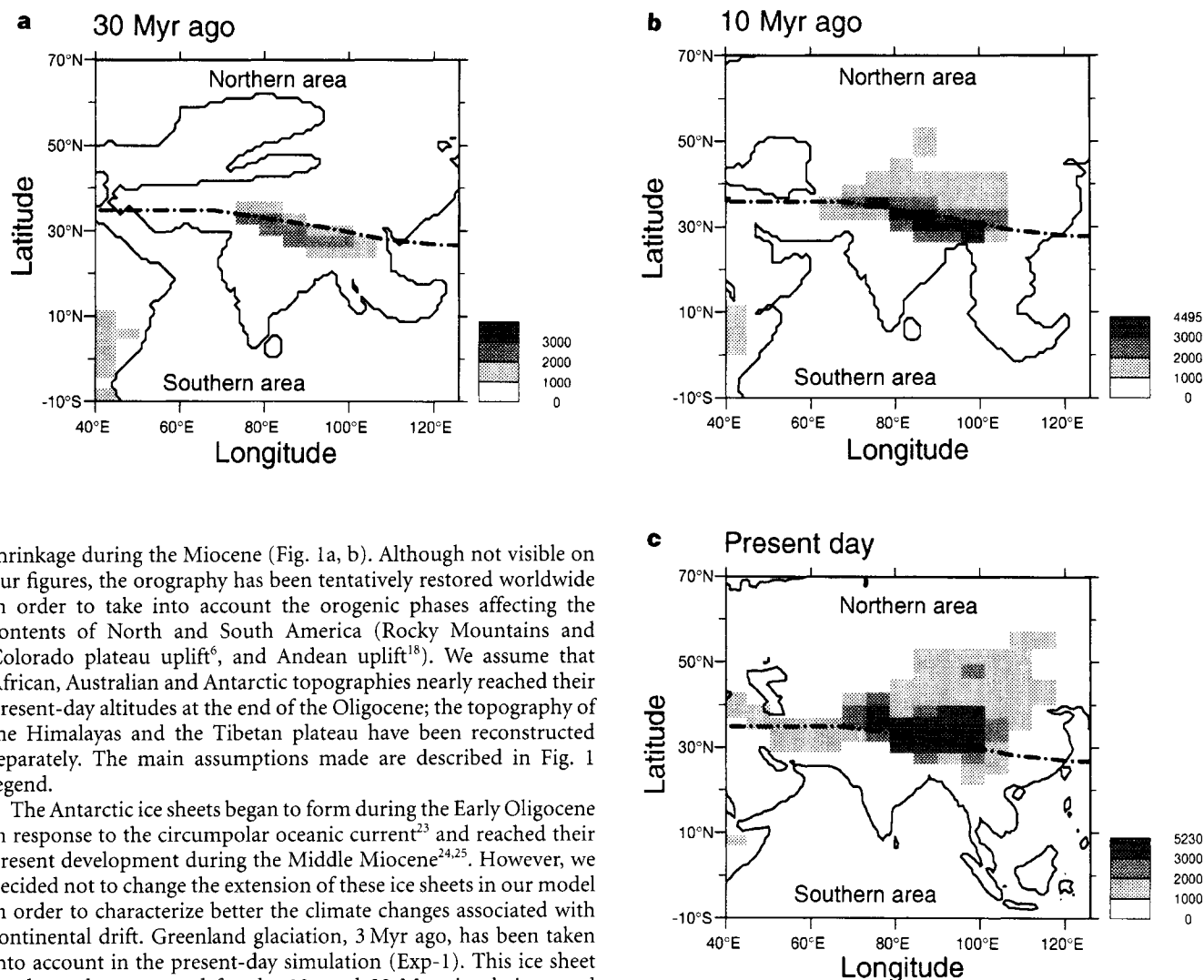


Figure 1 Eurasian palaeogeography and topography. **a**, Early Oligocene (simulation Exp-3); **b**, Middle-to-Late Miocene (Exp-2); **c**, present day (Exp-1). The shaded areas represent the relief interpolated into the AGCM grid point; see shading scale at lower right of each panel, which gives elevation in m above sea level. A dash-dotted line separates the northern and southern areas. A chain of moderate elevation corresponding to the proto-Himalayas may have existed during the Oligocene while the Tibetan plateau was most probably a peneplain covered with subtropical vegetation³⁴ at low elevation (no more than 500 m). Geological evidence then indicates a major Lower Miocene uplift in the region of the Gangdese belt^{43–45}, which is marked on our 10-Myr map (**b**) by higher elevation of the Himalayas. The Late Miocene stage may represent an intermediate phase of the Tibetan uplift evolution. We have introduced a gradient in the elevation of Tibet (lower altitude to the north, higher to the south) linked to its progressive build-up along its southern edge and a possible intracontinental subduction of Mongolia under it⁴⁶. The ultimate Himalayan uplift probably took place since the Pliocene. Besides, the tectonic evolution of Eurasia (linked to the general collision context from east to west of this plate) contributes to the progressive isolation of the intracontinental Paratethys sea⁴⁷, the renewal of water being realized by fluvial drift. The deposition of detrital sediments have possibly led to the progressive shrinkage of this marine domain during the Upper Miocene^{31,48}. Other orogenic phases (not shown) have affected different parts of the Earth since the Oligocene. In the western part of the North American continent, important uplifts took place affecting mainly the Rocky Mountains and the Colorado plateau after the Miocene⁹. In the South American continent, Andean uplift⁴⁹ occurred in several pulses, mainly between 26 and 6 Myr ago approximating a gradual uplift since the Oligocene. We assume that African, Australian and Antarctic topographies reached their present-day altitudes at the end of the Oligocene.

shrinkage during the Miocene (Fig. 1a, b). Although not visible on our figures, the orography has been tentatively restored worldwide in order to take into account the orogenic phases affecting the contents of North and South America (Rocky Mountains and Colorado plateau uplift⁶, and Andean uplift¹⁸). We assume that African, Australian and Antarctic topographies nearly reached their present-day altitudes at the end of the Oligocene; the topography of the Himalayas and the Tibetan plateau have been reconstructed separately. The main assumptions made are described in Fig. 1 legend.

The Antarctic ice sheets began to form during the Early Oligocene in response to the circumpolar oceanic current²³ and reached their present development during the Middle Miocene^{24,25}. However, we decided not to change the extension of these ice sheets in our model in order to characterize better the climate changes associated with continental drift. Greenland glaciation, 3 Myr ago, has been taken into account in the present-day simulation (Exp-1). This ice sheet has been thus removed for the 10- and 30-Myr simulations and replaced by a palaeoelevation deduced from isostatic rebound.

The insolation at the top of the atmosphere (orbital parameters) and CO₂ partial pressure are kept at their present-day values as we only wanted to trace the effect of palaeogeography on climate changes. (Moreover, computation of orbital parameters is not possible for times earlier than 5 Myr ago²⁶.) The partial pressure of CO₂ 30 Myr ago may have been 1.5–2 times higher than the present-day value²⁷. As shown by doubling-CO₂ experiments using the same model²⁸, this change could result in a temperature increase, especially at high latitudes. It may also have an effect on monsoon precipitation¹¹ but should not change our major conclusion about the respective effects of the Paratethys shrinkage and the Tibetan plateau uplift.

The sea surface temperatures (SSTs) are prescribed for this first set of experiments, using a 10-year average of present-day SSTs²⁹. When grid points pass from terrestrial to oceanic, we take the SST value of the nearest oceanic grid point at the same latitude. For the Paratethys, SSTs similar to those of seas present in the same region (for example, the Caspian Sea) are used. To quantify the changes in simulated climates that occur when we compute SSTs with a slab ocean, we performed a supplementary set of numerical experiments at lower resolution (48 points in longitude and 36 in sine of latitude) coupling the AGCM with a mixed-layer ocean of constant depth (50 m). For each period, we prescribed an oceanic heat transport deduced from the control run when accounting for continental drift. These experiments last 25 model years, 15 years being necessary to reach equilibrium in the coupled model. Results are averaged over the last 10 years of the simulation.

Climate change over Eurasia

We will analyse separately the evolution of Eurasian climate within two geographical regions, a northern and a southern part (Fig. 1). **Northern area.** During most of the Oligocene time, the Paratethys sea still covers a large area of central Eurasia (Fig. 1a). Its presence crucially influences the climate; it damps the seasonal thermal contrast, owing to its high water heat capacity, and provides an intense source of water vapour. The annual thermal amplitude over this sea does not exceed 17°C. The Paratethys maintains winter temperatures between 5 and 10°C in central Asia and between -10 and 0°C in Siberia. As a consequence, the Asian high-pressure area is restricted to China, and a stationary wave, forced by cyclonic activity, develops over the Paratethys. Precipitation is increased

between 45°N and 60°N by up to 900 mm yr⁻¹; there is a maximum of 1,500 mm yr⁻¹ upstream of this stationary wave where a trough is located (over Russia) and a drier area over northern China, with less than 500 mm yr⁻¹, downstream. These results are different from those that have been obtained using the 'no-mountain' simulations^{1-3,6} where a mainly zonal atmospheric circulation was found, with the maximum of precipitation centred on the fastest axis of wind in the upper troposphere in association with the polar front in the middle latitudes³. These differences highlight the major role of the Paratethys sea which acts as a thermal regulator, allowing the development of an oceanic climate over the main part of northern Eurasia.

The shrinkage of the Paratethys sea from the Oligocene to the

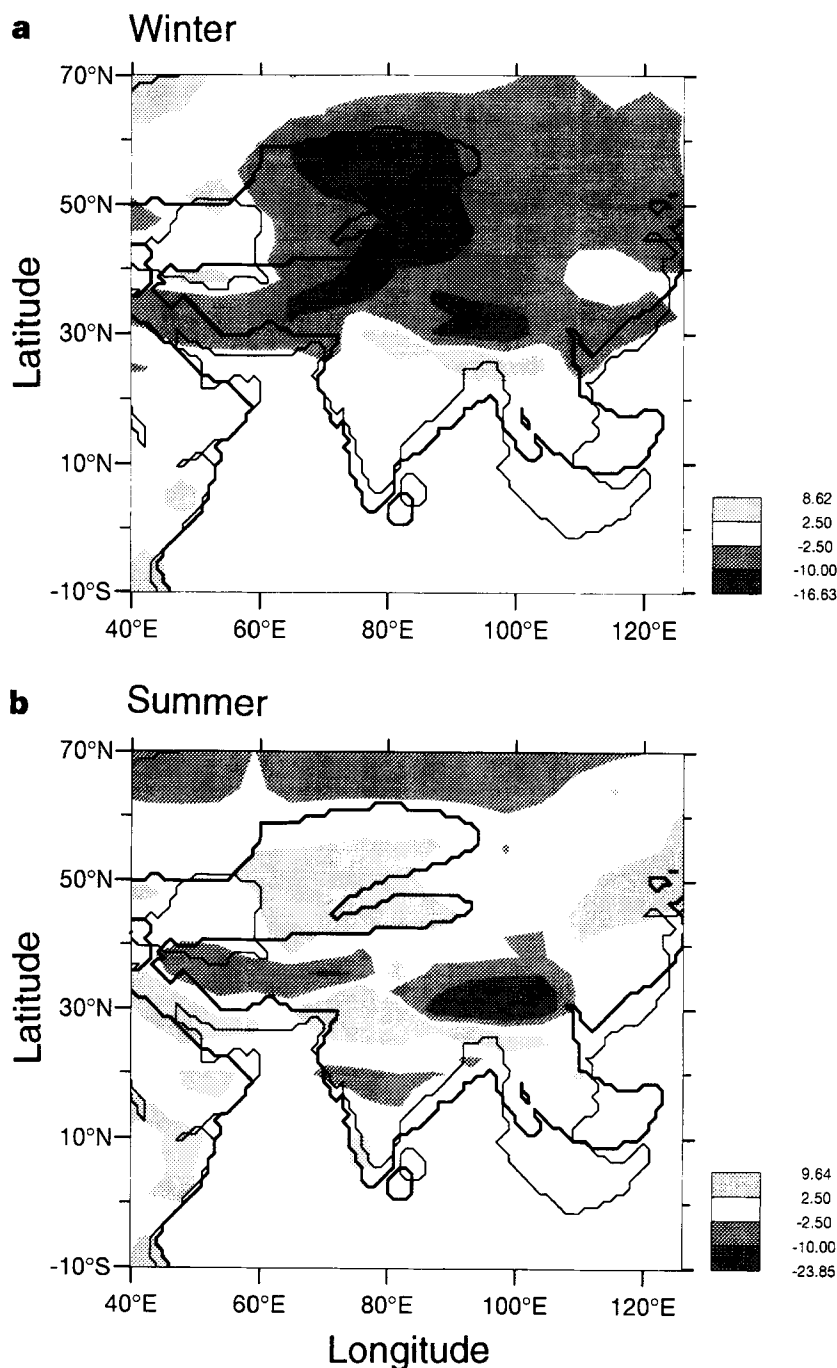


Figure 2 Mean temperature differences (°C; see shading scales) between the Early Oligocene and the Middle-to-Late Miocene (Exp-3 - Exp-2) in: **a**, winter (December to February); **b**, summer (June to August). The thick line (thin line)

represents the Eurasian palaeogeography of the Early Oligocene (Middle-to-Late Miocene).

Middle-to-Late Miocene changes the climate towards more continental conditions. The mean temperature differences between the Oligocene and the Miocene (Fig. 2a) show a cooling during winter of about 10 °C in Central Asia while the summer warming reaches 4 °C (Fig. 2b). The annual thermal amplitude then exceeds 30 °C. The winter precipitation on eastern Eurasia decreases because the most important source of moisture has disappeared. The shrinkage of the Paratethys also influences the large-scale atmospheric circulation. It should induce a more zonal atmospheric circulation over this region, but the weakening of this planetary wave is compensated by the development of a new planetary oscillation due to the Tibetan plateau uplift. Downstream of this stationary wave, a sheltered area in central Asia experiences a desertification. The plateau of Tibet itself becomes colder during all the seasons, due mainly to the uplift (Fig. 2a, b). In winter, a high-pressure cell is shifted westward and centred over Siberia, hence cold air masses slide down over the northern margin of the plateau and strengthen the continental climate. The presence of the Himalayan mountain range limits the outflow of cold air towards the Indian subcontinent.

The continentalization of climate is further intensified between Middle-to-Late Miocene and the present day. The final shrinkage of the Paratethys sea (Fig. 1c) allows for the development of the

Siberian high-pressure cell in winter, with a northward drift due to Tibetan uplift and an enhanced annual thermal amplitude. Both the Himalayan and Tibetan uplifts strengthen the winter ridge, and thus the desertification of central Asia and regions of the Middle East. Effects resulting from both the Tibetan uplift and Paratethys retreat also occur in summer: during the Oligocene, westerly winds blow over the Paratethys sea, but after the uplift of the Tibetan plateau enhanced winds are diverted around the high topography, and northerly winds blow above central Asia. Drier climates spread over Arabia and northeastern Africa in response to the progressive closure of the Tethys seaway^{30–32}. Despite the scarcity of reliable and representative palaeobotanical data for these periods, it is possible to use them to compare the major climate changes simulated by the model. The Oligocene environment in Kazakhstan (broad-leaved forests and swamps indicating a wet climate³³) is mainly replaced by dry biomes after the Late Miocene. In Siberia, the vegetation consists of swampy coniferous and broad-leaved forests during the Oligocene, evolving towards small-leaved taxa and conifers during the Late Miocene³³. These data confirm the atmospheric circulation changes found in our simulations, and demonstrate an important increase of seasonality leading to a more continental and colder climate (strong cooling in winter and small warming in

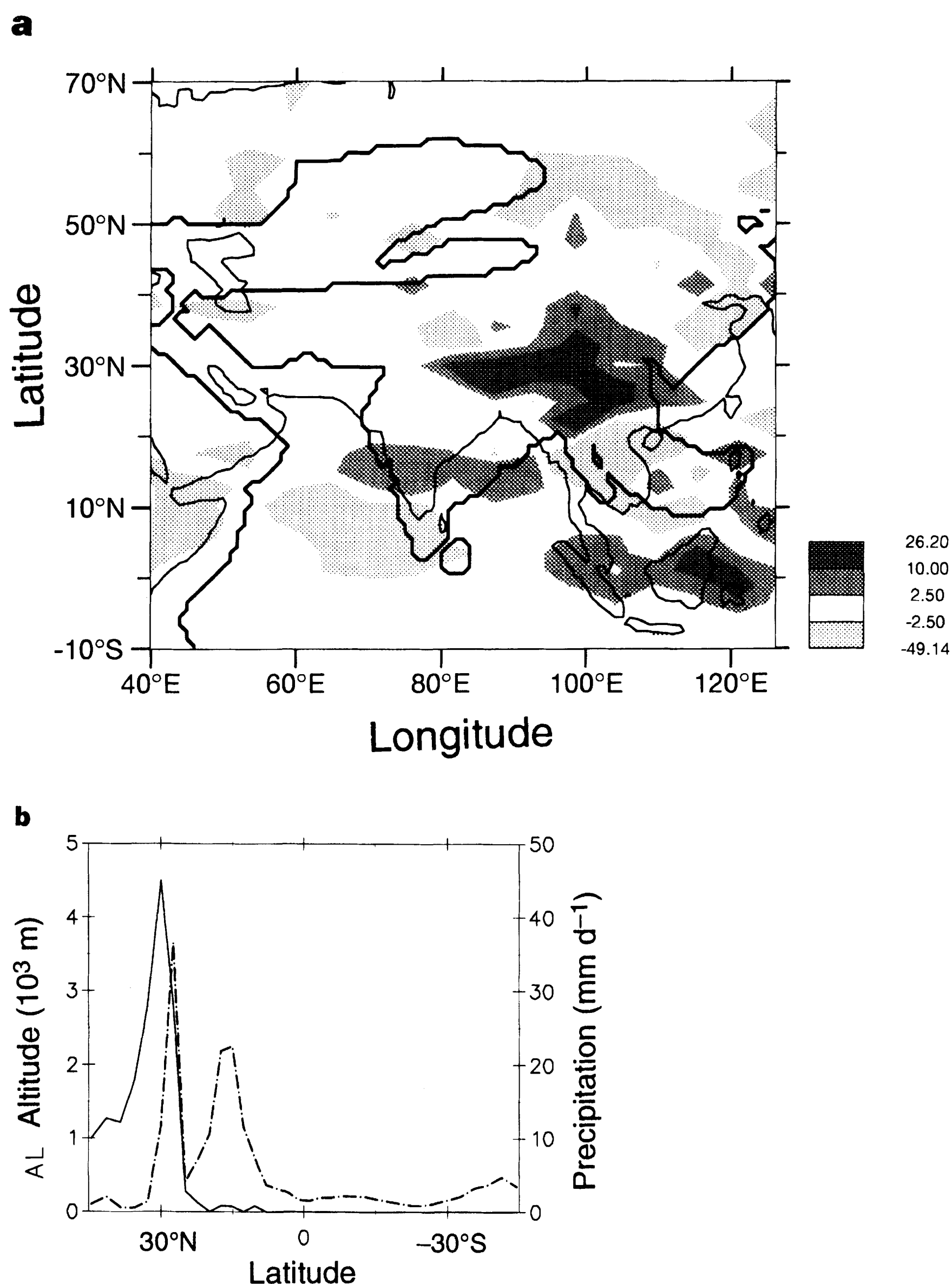


Figure 3 a, Mean precipitation differences (mm d^{-1} ; see shading scale) for summer (June to August) over Eurasia between the present day and the Early Oligocene (Exp-1 – Exp-3). The thick line (thin line) represents the palaeogeography of the Early Oligocene (present day). **b**, Zonal mean of summer precipitation (right-hand scale) and topography (left-hand scale) between 90°E and 100°E. The continuous (dash-dotted) line represents the elevation (precipitation).

summer). In central Tibet, palaeobotanic data show evergreen broad-leaved forests during the Oligocene, and emergence of alpine coniferous deciduous forests during the Miocene³⁴. However, these changes in vegetation are consistent with the predicted changes in temperature. There are also discrepancies between data and model results: palaeobotanic studies^{33,35} indicate the presence of forests close to the Arctic Ocean, whereas we find a polar climate in this area. The over-estimation of the pole–Equator thermal gradient in our simulation can be attributed to the atmospheric CO₂ level, which has been kept at its present value; a CO₂ enhancement would increase high-latitude temperatures.

Southern area. The monsoon is predicted to be weaker during Oligocene times than it is now (Fig. 3a). This is mainly due to the extension of the Paratethys sea which damps the seasonal cycle and weakens the summer thermal low. Westerly winds carry wet air masses from the Arabian sea over southern India and Indochina along the southern margin of the proto-Himalayan range without producing significant precipitation. A consequence of the Paratethys shrinkage is the increase of the summer temperature in central Eurasia (Fig. 2b) and the enhancement of the summer convergence of air towards the continental interior. During the Miocene, the first uplift phase of the Himalayas and the Tibetan

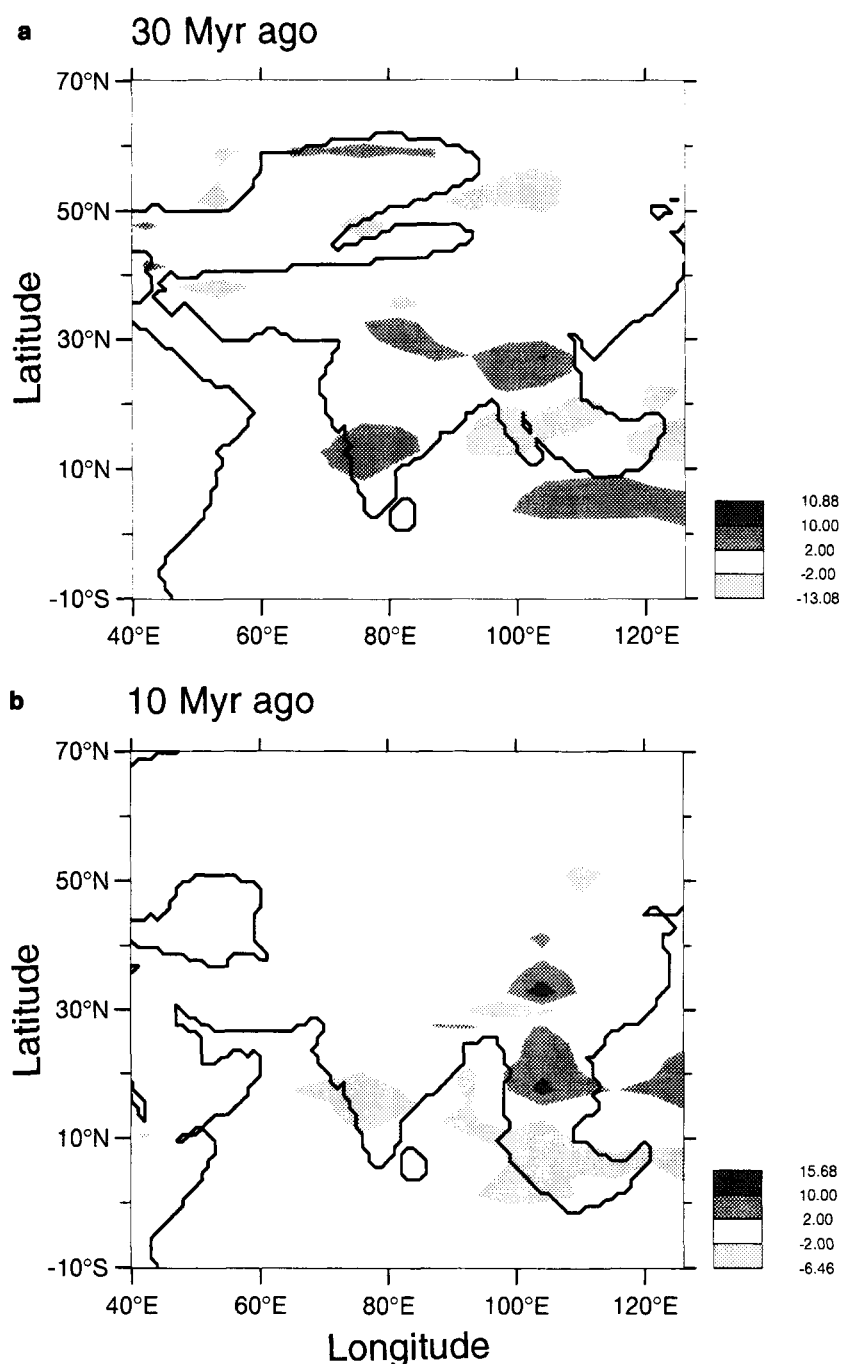


Figure 4 a. Mean precipitation differences (mm d^{-1} ; see shading scale) for summer (June to August) between the Oligocene experiments in response to the Tibetan uplift (Exp-4 – Exp-3). The continuous line represents the Oligocene palaeogeography. **b.** Mean precipitation differences (mm d^{-1}) for summer (June

to August) between Miocene experiments in response to the Tibetan uplift (Exp-5 – Exp-2). The continuous line represents the Middle-to-Late Miocene palaeogeography.

plateau enhances the vertical motion of air masses above them. The extended low-pressure cell over the Indogangetic basin and Eurasia deepens, leading to an increased convergence of the atmospheric flow towards the centre of the continent above northeastern India. In response to this stronger advection, wind speed increases over the Arabian sea by 75% between Oligocene and Miocene times and only by 30% between Miocene time and the present day. These last results can be correlated during the Middle-to-Late Miocene with a rapid development of planktonic foraminifers¹² and radiolarians^{35,36} in the Arabian Sea (in response to stronger upwellings). Air masses are compelled to rise, and water to condense, over the southern flank of

the Himalayas producing a high rate of precipitation; air masses are generally drier when sliding down the opposite side towards Tibet (Fig. 3b). Thus the Himalayan range produces a 'rain-shadow effect' over the plateau of Tibet and hence a decrease of precipitation over the western plateau (Fig. 3a) which is consistent with palaeodata³⁴. Because of a crude palaeogeography approximation, this precipitation pattern could not be simulated in previous experiments^{6,9-11}. The eastern part of the plateau is not subject to the same decrease of precipitation because of the smaller size of its mountain range; this allows the penetration of wet air masses into eastern Tibet via an easterly flow. The increase of monsoon rainfall has been recorded

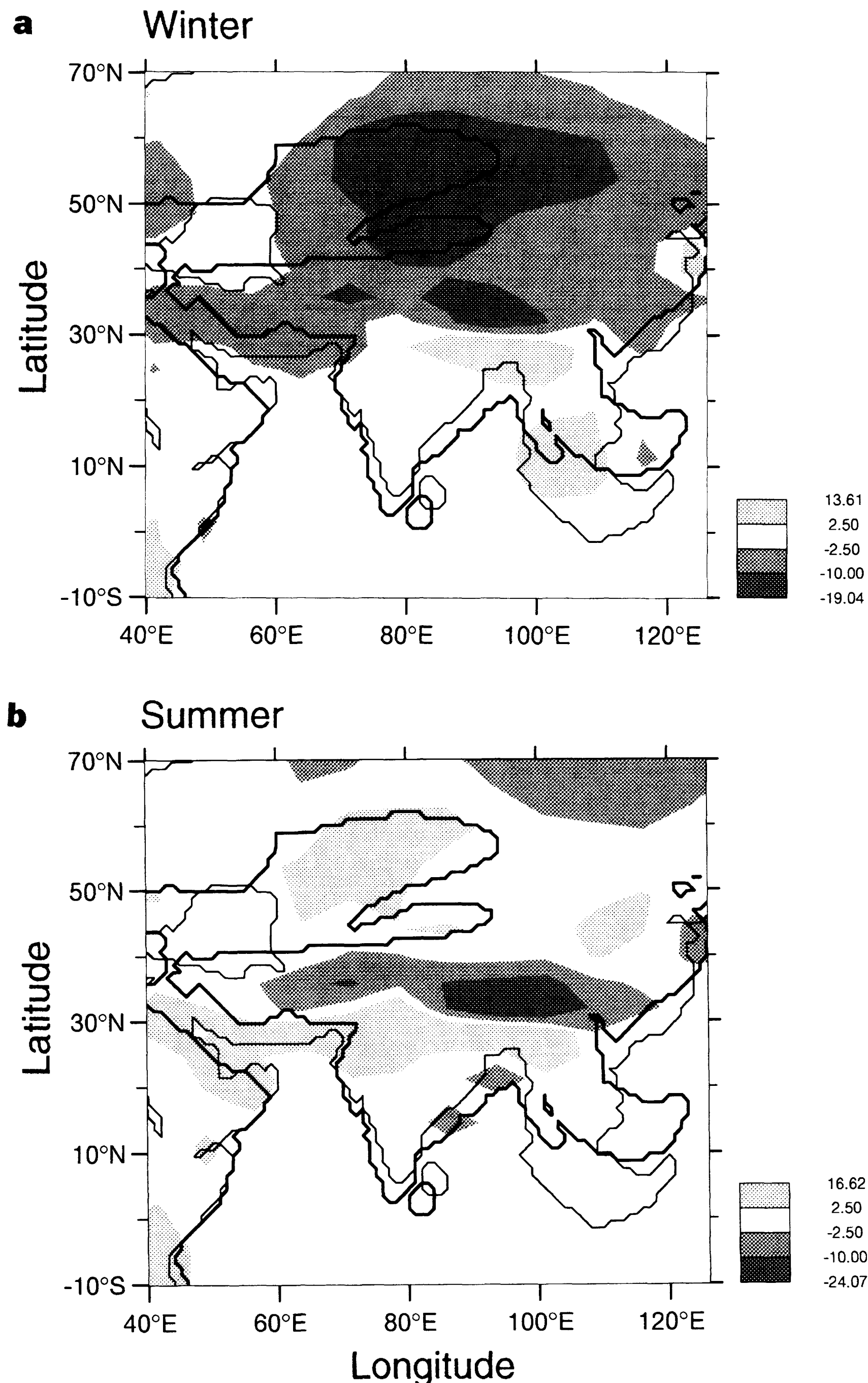


Figure 5 Mean temperature differences (°C; see shading scales) between the Early Oligocene and the Middle-to-Late Miocene using the Laboratoire de Météorologie Dynamique (LMD) AGCM coupled with a slab ocean (depth

50 m). **a**, Winter (December to February); **b**, summer (June to August). The thick line (thin line) represents the Eurasian palaeogeography of the Early Oligocene (Middle-to-Late Miocene).

in Bengal fan sediments²⁸ where a high rate of sediment delivery has been observed during the Middle-to-Late Miocene; this was followed by a major geochemical transition in sediment assemblage. Moreover, since the Early Miocene, most of the sediments deposited in the Bengal fan come essentially from the Himalayas rather than from the Tibetan plateau, which is consistent with the predicted high rate of precipitation on the southern flank of Himalayas for our 10-Myr experiment.

Continental drift also plays a role in the redistribution of monsoon precipitation. Indeed, India continuously drifts northwards during the period between 30 and 10 Myr ago with an average speed of 4 cm yr^{-1} , corresponding to a northward motion of 500 km. During the Oligocene, the southern part of India is located below the inter-tropical convergence zone (ITCZ) with annually distributed precipitation and migrates northwards during the Miocene leading to drier spring and autumn seasons. Over the middle part of India, the monsoon represents less than 60% of the rainfall in the Oligocene, and nearly 80% during the Miocene, leading also to a more contrasted climate with longer dry periods. The palaeobotanic data confirm this evolution. Tropical vegetation disappears progressively during the Miocene and is replaced by plants characteristic of more arid regions, and a savannah belt composed mainly of xerophytic plants develops over eastern Africa and western India^{37,38}. Pedogenic carbonates in Siwalik basin in Pakistan also suggest a climate shift around 7–8 Myr ago^{39,40}. The $\delta^{13}\text{C}$ record shows an ecological transition from C3 to C4 type vegetation with more seasonal rainfall¹². Tropical biomes (rain-forest plants) move northwards and find a shelter on the southern side of the Himalayas where summer precipitation and hydrological contributions sustain a high level of moisture (Fig. 3a, b).

Indochina's southward motion induces an opposite precipitation-pattern evolution to that of India. During the Oligocene, the monsoon represents around 70% of annual precipitation. During the Miocene, the northern part of Indochina remains affected by monsoon precipitation (with an increase located near the high Asian topography), whereas the southern part (Fig. 3a), located over equatorial latitudes, becomes sensitive to annually distributed ITCZ precipitation. Consequently, a wet climate remains over this latter

area. Palaeodata confirm this result: a monsoon palaeoenvironment has been found in Thailand⁴¹ since the Early Miocene and also in southern China since the Oligocene⁴².

Sensitivity experiments

Here we investigate the relative effect of the Paratethys retreat and the Tibetan plateau uplift. All the sensitivity experiments last 15 model years; Student's *t*-tests show that the changes in precipitation described below are statistically significant ($>95\%$).

We first compare (Fig. 4a) the precipitation between the Early Oligocene reconstructions with (Exp-3) and without (Exp-4) the Paratethys sea. The enhancement of continental warming due to the disappearance of the Paratethys sea increases the thermal gradient between central Asia and the Indian Ocean, leading to a large increase in monsoon precipitation over the southeastern flank of the Himalayas and the middle part of India. We then compare (Fig. 4b) the precipitation between Miocene palaeogeography with the Tibetan plateau at 3,500 m (Exp-2) and the same palaeogeography with the plateau reduced to 900 m (Exp-5). In contrast to the Exp-3/Exp-4 comparison, we find an appreciable increase only over the easternmost flank of the southern Himalayas and within Indochina (Fig. 4b). Figure 4a thus suggests that much of the rainfall increase between 30 Myr and the present day is due to Paratethys retreat.

However, our results may be biased by the use of prescribed SSTs at their present-day values. To quantify the sensitivity to SSTs, we performed a second set of numerical experiments in which the AGCM was coupled with a slab ocean. We found that enhancement in seasonality between 30 and 10 Myr, and between 10 Myr and the present day, that we observed with fixed SSTs (Fig. 2a) is slightly increased with computed SSTs.

In Fig. 5 we plot the temperature differences between 30 and 10 Myr ago, for winter and summer. (The comparison between Figs 5 and 2 shows similar changes in temperatures for both seasons.) In winter (Fig. 5a), the cooling area is larger than for the fixed SST experiment, with a temperature decrease over the Arabian sea and a more pronounced cooling over the northern part of the Paratethys sea. In summer (Fig. 5b), the warming is 5°C in the area of Paratethys shrinkage. (The lower resolution used in these simula-

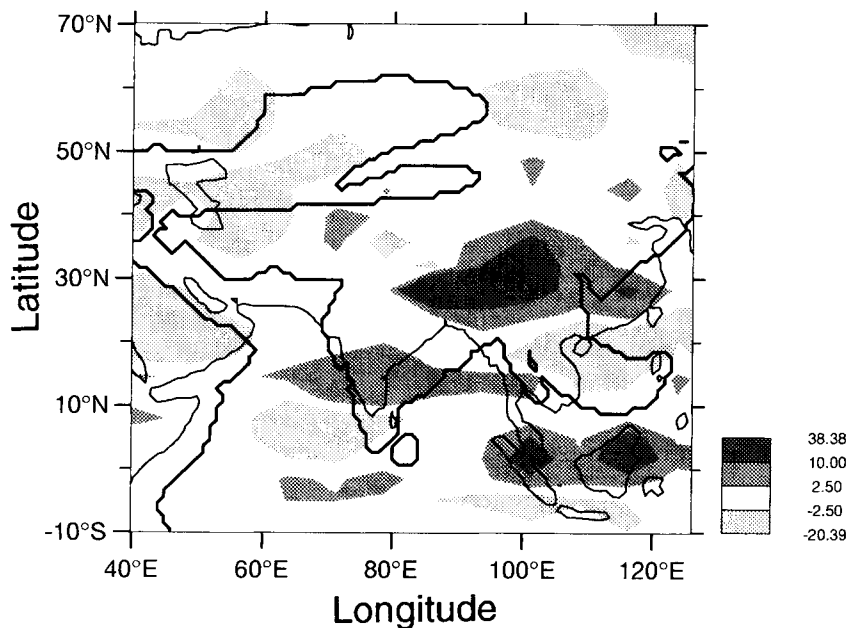


Figure 6 Mean precipitation differences (mm d^{-1} ; see shading scale) for summer (June to August) between the Early Oligocene and the present day using the LMD

AGCM coupled with a slab ocean (depth 50 m). The thick line (thin line) represents the palaeogeography of the Early Oligocene (present day).

tions has the consequence of shifting northwards the maximum elevation of the Himalayas by one grid point between 10 and 30 Myr which induces a warming along this mountain range.) Figure 6 shows the differences in summer precipitation between 30 Myr ago and the present day, obtained with computed SSTs. Once more, comparing these results with those obtained with prescribed SSTs (Fig. 3a), we see that the enhanced monsoon precipitation has a similar location and amplitude despite a small difference in its northward extent which is due to the coarser resolution used (lower elevation of the Himalayas). In contrast, precipitation is strongly modified over Indochina and India, mainly due to changes in the simulation of the ITCZ between fixed and computed SSTs. Overall, the effect of coupling our AGCM with a slab ocean is to damp and shift northwards the ITCZ, which modifies the precipitation pattern over Indochina and southern India.

Towards a more realistic picture

Previous sensitivity experiments on plateau uplift^{6,9–11} using AGCMs have revealed major trends in climate changes that occurred over Eurasia since the Indian collision, namely the enhancement of monsoon rainfall and the cooling of Eurasia. Our new simulations, using more realistic paleogeographical reconstructions for two key periods, the Early Oligocene and the Middle-to-Late Miocene, reveal clearly that the Paratethys shrinkage has equally drastic consequences for climate over central Asia and eastern Europe during this period, driving the climate change from temperate to continental conditions. In contrast with previous simulations, taking into account different orographies and uplift rates for the Tibetan plateau and the Himalayas, we show that between these periods monsoon rainfall increased drastically over the southern flank of Himalayas, whereas the central and northern part of Tibet remained dry. This evolution is in agreement with many paleoclimate indicators.

Another important result is that the respective northward and southward motions of India and Indochina drastically modify the precipitation pattern over these regions. However, these results are sensitive to SST. More quantitative data and sensitivity experiments regarding atmospheric CO₂ concentrations would certainly be useful to enhance the reliability of simulations of climate change in Eurasia between 30 Myr and the present day: nevertheless, the Paratethys shrinkage appears to play a major role in the increase of monsoon precipitation and in the cooling of central Eurasia. □

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- Manabe, S. & Terpstra, J. B. The effects of mountains on the general circulation of the atmosphere as identified by numerical experiments. *J. Atmos. Sci.* **31**, 3–42 (1974).
- Manabe, S. & Broccoli, A. J. Mountains and arid climate of middle latitudes. *Science* **247**, 192–195 (1990).
- Broccoli, A. J. & Manabe, S. The effect of orography in midlatitude northern hemisphere dry climates. *J. Clim.* **5**, 1181–1201 (1992).
- Hahn, D. G. & Manabe, S. The role of mountains in the south Asian monsoon circulation. *J. Atmos. Sci.* **32**, 1515–1541 (1975).
- Barron, E. J., Thompson, S. L. & Hay, W. W. Continental distribution as a forcing factor for global-scale temperature. *Nature* **310**, 574–575 (1984).
- Kutzbach, J. E., Guetter, P. J., Ruddiman, W. F. & Prell, W. L. Sensitivity of climatic uplift in Southern Asia and in the American West: Numerical experiments. *J. Geophys. Res.* **94**, 18393–18407 (1989).
- Kutzbach, J. E. & Ziegler, A. M. Simulations of late Permian climate and biomes with an atmosphere-ocean model: comparisons with observations. *Phil. Trans. R. Soc. Lond. B* **341**, 327–340 (1993).
- Ruddiman, W. F., Prell, W. L. & Raymo, M. E. Late Cenozoic uplift in the Southern Asia and the American West: rationale for General Circulation Modeling Experiment. *J. Geophys. Res.* **94**, 18379–18391 (1989).
- Ruddiman, W. F. & Kutzbach, J. E. Forcing of the late Cenozoic uplift northern hemisphere climate by plateau uplift in the Southern Asia and American West. *J. Geophys. Res.* **94**, 18409–18427 (1989).
- Kutzbach, J. E., Prell, W. L. & Ruddiman, W. F. Sensitivity of Eurasian climate to surface uplift of Tibetan plateau. *J. Geol.* **101**, 177–190 (1993).
- Prell, W. L. & Kutzbach, J. E. Sensitivity of the Indian monsoon to forcing parameters and implications for its evolution. *Nature* **360**, 647–652 (1992).
- Prell, W. L., Murray, D. W., Clemens, S. C. & Anderson, D. M. in *Synthesis of Results from Scientific Drilling in the Indian Ocean* (ed. Duncan, R. A.) 447–470 (Geophys. Monogr. 70, Am. Geophys. Union, Washington DC, 1992).

- Harzallah, A. & Sadourny, R. Internal versus SST-forced atmospheric variability as simulated by an atmospheric general circulation model. *J. Clim.* **8**, 474–495 (1995).
- Royer, J. Y. & Sandwell, D. T. Evolution of the Eastern Indian Ocean since the Late Cretaceous: constraints from Geosat altimetry. *J. Geophys. Res.* **94**, 13755–13782 (1989).
- Nürnberg, D. & Müller, R. D. The tectonic evolution of the South Atlantic from Late Jurassic to Present. *Tectonophysics* **181**, 27–33 (1991).
- Olivet, J. L., Bonnin, J., Beuzart, P. & Auzende, J. M. *Cinématique de l'Atlantique Nord et Central* (Cent. Nat. Explor. Océans, Brest, 1984).
- Besse, J. & Courtillot, V. Paleogeographic maps of the continents bordering the Indian Ocean since the early Jurassic. *J. Geophys. Res.* **93**, 11791–11808 (1988).
- Besse, J. & Courtillot, V. Revised and synthetic apparent polar wander paths of the African, Eurasian, North-American and Indian plates, and true polar wander since 200 Ma. *J. Geophys. Res.* **96**, 4029–4050 (1991).
- Yang, Z. & Besse, J. Paleomagnetic study on Permian and Mesozoic sedimentary rocks from North Thailand supports the extrusion model for Indochina. *J. Geophys. Res.* **117**, 525–552 (1993).
- Chen, Y. *et al.* The configuration of Asia prior to the collision of India: Cretaceous paleomagnetic constraints. *J. Geophys. Res.* **98**, 21927–21941 (1993).
- Biais, A., Patriat, P. & Tappinier, P. Updated interpretation of the magnetic anomalies and seafloor spreading stages in the south China Sea. *J. Geophys. Res.* **98**, 6299–6328 (1993).
- Dercourt, J., Ricou, L. E. & Vrielynck, B. (eds) *Atlas Tethys Palaeoenvironmental Maps* 1–307 (Gauthier-Villars, Paris, 1993).
- Kravov, D. D. & Verbitsky, M. Ya. Causes of Antarctic glaciation in the Cenozoic. *Quat. Res.* **15**, 1–17 (1981).
- Shackleton, N. J. & Kennett, J. P. Paleotemperature history of the Cenozoic and the initiation of Antarctic Glaciation: oxygen and carbon isotopes analyses in DSDP Site 277, 279, and 291. *Init. Rep. DSDP* **29**, 743–756 (1975).
- Barrett, P. J., Elston, D. P., Harwood, D. M., McKelvey, B. C. & Webb, P. N. Mid-Cenozoic record of glaciation and sea-level change on the margin of the Victoria Land Basin. *Geology* **15**, 634–637 (1987).
- Lasker, J. Secular evolution of the solar system over 10 million years. *Astron. Astrophys.* **98**, 341–362 (1988).
- Berner, R. A., Lasaga, A. C. & Garrels, R. M. The carbonate-silicate geochemical cycle and its effects on atmospheric carbon dioxide over the past 100 million years. *Am. J. Sci.* **283**, 641–683 (1983).
- Le Treut, H., Li, Z. X. & Forrichon, M. Sensitivity of the LMD general circulation model to greenhouse forcing associated with two different cloud water parameterizations. *J. Clim.* **7**, 1827–1841 (1994).
- Joussaume, S. & Taylor, K. in *Proc. 1st Int. AMIP Sci. Conf. WRCP 92* (ed. Gates, L. W.) 425–430 (TD No. 732, WMO, Geneva, 1995).
- Biju-Duval, B., Delcourt, J. & LePichon, X. From the Tethys Ocean to the Mediterranean Sea: a plate tectonic model of the evolution of the western alpine system. 143–164 (Technip, Paris, 1976).
- Orszag-Sperber, F. *et al.* in *Atlas Tethys Palaeoenvironmental Maps* (eds Dercourt, J., Ricou, L. E. & Vrielynck, B.) 243–258 (Gauthier-Villars, Paris, 1993).
- Ricou, L. E. Tethys reconstructed: plates, continental fragments and their boundaries since 260 Ma from Central America to South-eastern Asia. *Geodinamica Acta (Paris)* **7**, 169–218 (1994).
- Zubakov, V. A., Borzenkova, I. I. *Global Palaeoclimate of the Late Cenozoic* (Elsevier, Amsterdam, 1990).
- Ren, X. in *Geological and Ecological Studies of Qinghai-Xizang Plateau* (ed. Gordon, B.) 139–144 (Science Press, Beijing, 1981).
- Nigrini, C. & Caulet, J. P. Late Neogen radiolarian assemblages characteristics of Indo-Pacific areas of upwelling. *Micropaleontology* **38**, 139–164 (1992).
- Molnar, P., England, P. & Martinod, J. Mantle dynamics, uplift of the Tibetan plateau, and the Indian monsoon. *Rev. Geophys.* **31**, 357–396 (1993).
- Traverse, A. Response of world vegetation to Neogene tectonic and climatic events. *Alcheringa* **6**, 197–209 (1982).
- Wolfe, J. A. in *The Carbon Cycle and Atmospheric CO₂: Natural Variations Archean to Present* (eds Sundquist, E. T. & Broecker, W. S.) 357–375 (Am. Geophys. Union, Washington DC, 1985).
- Quade, J., Cerling, T. E. & Bowman, J. R. Development of Asian monsoon revealed by marked ecological shift during the latest Miocene in northern Pakistan. *Nature* **342**, 163–166 (1989).
- Cerling, T. E., Wang, Y. & Quade, J. Expansion of C₄ ecosystems as an indicator of global ecological change in the Miocene. *Nature* **361**, 344–345 (1993).
- Ducrocq, S., Chaimanee, Y., Suteethorn, V. & Jaeger, J. J. Ages and paleoenvironment of Miocene mammalian faunas from Thailand. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **108**, 149–163 (1994).
- Chenggao, G. & Renaut, R. W. The effect of Tibetan uplift on the formation and preservation of Tertiary lacustrine source-rocks in eastern China. *J. Paleolimnol.* **11**, 31–40 (1994).
- Copeland, P., Harrison, T. M., Kidd, W. S. F., Ronghua, X. & Yuquan, Z. Rapid early Miocene acceleration of uplift in the Gangdese belt, Xizang (southern Tibet), and its bearing on accommodation mechanisms of the Indian-Asia collision. *Earth Planet. Sci. Lett.* **86**, 240–252 (1987).
- Harrison, T. M., Copeland, P., Kidd, W. S. F. & Yin, A. Raising Tibet. *Science* **255**, 1663–1670 (1992).
- Richter, F. M., Lovera, O. M., Harrison, T. M. & Copeland, P. Tibetan tectonics from 40Ar/39Ar analysis of a single K-feldspar sample. *Earth Planet. Sci. Lett.* **105**, 266–278 (1991).
- Tappinier, P. *et al.* Active thrusting and folding in the Qilian Shan, and decoupling between upper crust and mantle in northeastern Tibet. *Earth Planet. Sci. Lett.* **97**, 382–403 (1990).
- Baldi, T. The terminal Eocene and Early Oligocene events in Hungary and the separation of an anoxic, cold Paratethys. *Eclogae Geol. Helv.* **30**, 1–27 (1984).
- Lorenz, C. *et al.* in *Atlas Tethys Palaeoenvironmental Maps* (eds Dercourt, J., Ricou, L. E. & Vrielynck, B.) 211–233 (Gauthier-Villars, Paris, 1993).
- Sébrier, M., Lavenu, A., Fornari, M. & Soulas, J. P. Tectonics and uplift in Central Andes (Peru, Bolivia and Northern Chile) from Eocene to present. *Geodynamica* **3**, 85–106 (1988).
- Masson, V. & Joussaume, S. Energetics of the 6000 BP atmospheric circulation in boreal summer, from large scale to monsoon areas: a study with two versions of the LMD AGCM. *J. Clim.* (in the press).

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Phenotype of mice lacking functional *Deleted in colorectal cancer (Dcc)* gene

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The *DCC* (Deleted in colorectal cancer) gene was first identified as a candidate for a tumour-suppressor gene on human chromosome 18q. More recently, *in vitro* studies in rodents have provided evidence that *DCC* might function as a receptor for the axonal chemoattractant netrin-1. Inactivation of the murine *Dcc* gene caused defects in axonal projections that are similar to those observed in *netrin-1*-deficient mice but did not affect growth, differentiation, morphogenesis or tumorigenesis in mouse intestine. These observations fail to support a tumour-suppressor function for *Dcc*, but are consistent with the hypothesis that *DCC* is a component of a receptor for netrin-1.

DCC (Deleted in Colorectal Cancer) encodes a group of structurally similar transmembrane proteins of relative molecular mass 175,000–190,000 (M_r 175–190K) with four immunoglobulin and six fibronectin type III repeats in their extracellular domains¹. *DCC* was first identified as possible tumour-suppressor gene on human chromosome 18q that is frequently subject to loss of heterozygosity (LOH) in colorectal tumours¹. LOH is a process often used by developing tumour cell clones to eliminate both copies of a tumour-suppressor gene². Initially, one gene copy is inactivated by mutation; thereafter the second, intact gene copy is discarded and often replaced by a duplicated copy of the mutant allele. During the development of about 30% of colorectal tumours, LOH of 18q occurs in a relatively early step during which small adenomas (polyps) progress to more dysplastic, larger, intermediate adenomas³. In the advanced adenomas—the immediate precursors of carcinomas—the incidence of 18q LOH approaches 65%^{1,3,4}.

Consistent with the possibility that 18q carries a colonic tumour-suppressor gene are chromosome transfer experiments demonstrating that one normal copy of chromosome 18 is sufficient to suppress the tumorigenicity of human colon carcinoma cell lines^{5,6}. However, the accumulated evidence has failed to implicate *DCC* conclusively as the colorectal tumour-suppressor gene on chromosome 18q. For example, analysis of the *DCC* alleles retained in human colonic tumours led to the discovery of mutant alleles in only 2 of 60 colonic tumours affected by 18q LOH⁷. An additional 12% of the tumours carry insertions in one of the *DCC* introns¹, but the effect of these insertions on *DCC* function is not known. Some have suggested, however, that mutations of *DCC* are not necessary to eliminate *DCC* function because *DCC* RNA, which is at the threshold of detection by the polymerase chain reaction (PCR) in normal colonic tissue¹, is either reduced or completely lost in colon cancer cell lines presumably by some means other than intragenic mutation^{8,9}.

In vitro experiments have also failed to provide conclusive proof that *DCC* is a tumour-suppressor gene. One study showed that high

levels of antisense *DCC* RNA could promote the tumorigenicity of Rat-1 cells¹⁰, but did not control for the possibility that the long-term culture of the transfected cells inadvertently selected for those with increased tumorigenicity. A second study showed that *DCC* overexpression in immortalized human keratinocytes caused poor growth *in vitro* and reduced tumour growth *in vivo*¹¹ but did not exclude the possibility that high levels of *DCC* protein, which is not normally expressed in these cells, may, as many ectopically expressed proteins do, exert nonspecific cytostatic effects on cells.

An unrelated line of investigation has suggested that *DCC* may function as a component of a receptor complex that mediates the effects of the axonal chemoattractant netrin-1 on commissural axons of the spinal cord of vertebrates¹². Commissural neurons differentiate in the dorsal portion of the spinal cord and their axons pioneer a ventrally directed circumferential pathway that leads them to a specialized group of cells termed floor plate cells at the ventral midline of the spinal cord¹³. During development of the nervous system, these axons are guided to the floor plate at least partly by the chemoattractant netrin-1, secreted by floor-plate cells^{13–17}. Netrin-1 is a member of a phylogenetically conserved family of laminin-related molecules that has been implicated in axonal guidance in *Caenorhabditis elegans*, *Drosophila melanogaster* and vertebrates^{15–21}. Loss-of-function phenotypes and genetic interactions in *C. elegans* and *Drosophila* have suggested that homologues of the *DCC* protein function in the response pathway of netrin family members^{22,23}. In vertebrates, there is a biochemical interaction between netrin-1 and *DCC*, and *DCC* has been implicated in mediating netrin-1 effects on spinal commissural axons *in vitro* through antibody perturbation studies¹², but the significance of this interaction for guidance of axons by netrin-1 *in vivo* has not been addressed.

To help elucidate the functions of the *DCC* gene, we have inactivated its homologue (*Dcc*) in the mouse genome through use of homologous recombination and have examined the effects of

this inactivation on both the intestine and the developing nervous system.

Inactivation of the mouse homologue of DCC

The *Dcc*^{X3} targeting vector (Fig. 1a) was introduced into 129/Sv D3 embryonic stem (ES) cells²⁴. Southern blot analysis demonstrated that three of the 200 gancyclovir- and G418-resistant ES cell clones screened had acquired a copy of the neomycin transferase (*neo*) gene in the mouse *Dcc* locus by homologous recombination (Fig. 1b). We term this mutant allele *Dcc*⁻. Mouse chimaeras generated from two of these *Dcc*^{+/-} ES cell clones transmitted the *Dcc*⁻ allele through their germ line. All neonatal mice homozygous for the *Dcc*⁻ allele appeared to be grossly normal at birth but died within 24 h. The *Dcc*^{-/-} neonatal mice exhibited striking behavioural phenotypes, including the inability to suckle, laboured respiration, abnormal body posture and abnormal limb flexion in response to pinch stimuli. Immunoblots of proteins extracted from the brains of the *Dcc*^{-/-} pups confirmed that the *Dcc*^{X3} mutation caused complete loss of DCC protein expression in the homozygous mutant mice (Fig. 1c).

No increase in tumour predisposition

To determine if and when *Dcc*^{+/-} mice develop tumours, we followed a cohort of nearly 200 129Sv/C57BL6 (129Sv/B6) F1

Dcc^{+/-} mice for 2 years. *Dcc*^{+/-} animals were monitored closely and screened at different ages for tumours in their gut and extra-intestinal tissues. Only three *Dcc*^{+/-} mice displayed signs of sickness, each being observed at about 18 months of age. One of these animals had six adenomas in its duodenum. The other two mice had brain neoplasms, one an astrocytoma, the other a meningioma.

To determine whether aged *Dcc*^{+/-} mice have an increased tumour predisposition compared to the *Dcc*^{+/+} mice, we did an autopsy on 98 129Sv/C57BL6 (129Sv/B6) F1 and F2 *Dcc*^{+/-} mice and 36 *Dcc*^{+/+} littermates, aged 1.5 to 2 years. In addition, the entire intestine of each animal was examined for the presence of adenomas. *Dcc*^{+/-} mice showed no significant increase in tumour predisposition when compared to the control *Dcc*^{+/+} mice (Table 1). Most of the neoplastic lesions found in both cohorts were detectable only by histological examination of tissues removed at the time of autopsy. No brain tumours were found in either the *Dcc*^{+/-} or the *Dcc*^{+/+} cohort. Southern blot analysis of genomic DNA prepared from tumours removed from 18-month-old heterozygotes indicated that both the wild-type and the mutant *Dcc* alleles had been retained (data not shown; our analytical procedures were readily able to detect loss of the wild-type *Dcc* alleles in adenomas of comparable size, as described below). We have also monitored cumulatively an additional 300 *Dcc*^{+/-} mice for signs of brain tumours or intestinal polyposis but none were observed.

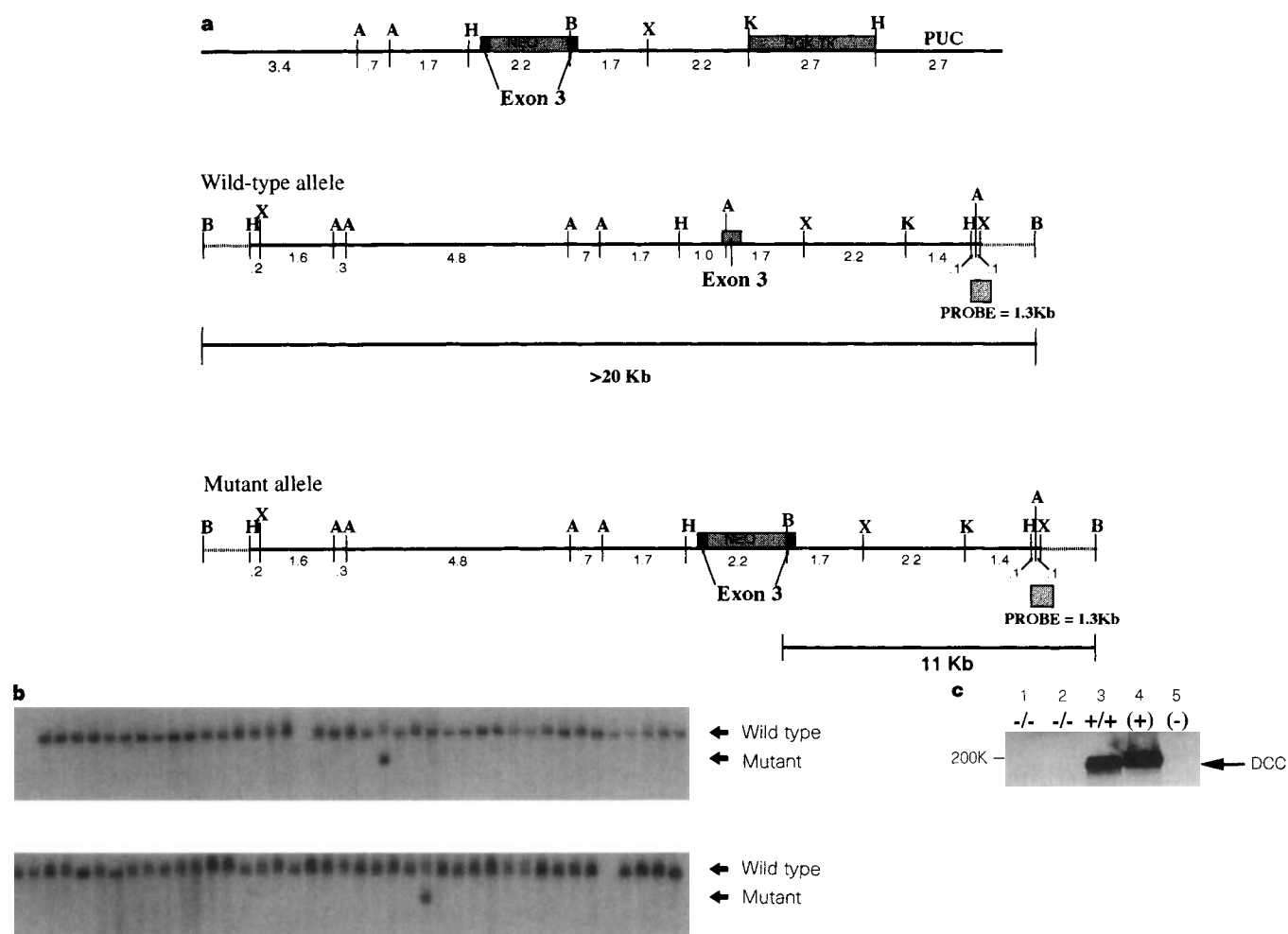


Figure 1 Construction of *Dcc*^{X3} mutation. **a**, *Dcc*^{X3} was constructed as described in the Methods. The HSV-tk and PMCI-*neo*-polA cassettes are transcribed in the same transcriptional orientation as the *Dcc* gene. (X, *Xba*I; H, *Hind*III; A, *Afl*II; B, *Bam*HI; K, *Kpn*I and N, *Not*I). **b**, Southern analysis of *Bam*HI-digested DNA from ES cell clones using a probe from intron 3 detects a *Dcc*^{X3}-specific 11-kb restriction fragment in addition to the >20 kb fragment corresponding to the wild-type *Dcc*

allele. **c**, DCC was immunoprecipitated from total brain protein (*Dcc*^{-/-} in lanes 1 and 2; *Dcc*^{+/+} in lane 3) using an antibody directed to the C terminus of DCC and western blotted as described previously⁵⁰. The full-length 190K DCC protein is indicated by arrow. Lane 4 represents a positive control and is a DCC-deficient human cell line transfected with a DCC-expressing construct and lane 5 is a negative control and is the same cell line before transfection⁵⁰.

Although germline inactivation of a *Dcc* gene copy did not result in an increased tumour predisposition, it seemed possible that loss of both gene copies would affect the progression of intestinal adenomas initiated by prior inactivation of the *Adenomatous polyposis coli* (*Apc*) gene. Indeed, LOH of 18q in human colonic adenomas correlates with an increase in their size and morphological changes such as acquisition of a more dysplastic phenotype^{3,4,25}, and some studies have linked the loss of *Dcc* expression to the appearance of adenomas that are of a more villous rather than tubular appearance²⁶. To test effects on tumour progression, we introduced the null *Dcc* allele into the germ line of the *Min* mouse²⁷ that carries a mutant *Apc* allele (*Apc*^{Min}) in its germ line that predisposes it to develop small intestinal and colonic adenomas^{27–29}.

Molecular genetic linkage and syntenic analyses have mapped the *Dcc* gene to mouse chromosome 18 within 30 cM from the *Apc* gene^{30,31}. Previous studies had demonstrated that nearly all adenomas in intestines of *Apc*^{Min/+} mice lose not only the wild-type allele of *Apc* but also the entire chromosome 18 harbouring the wild-type *Apc* allele³². This loss affects any heterozygosity that may exist at the *Dcc* locus, which happens to be located on chromosome 18³¹. We took advantage of this linkage by introducing the mutant *Dcc* allele into the chromosome carrying the *Apc*^{Min} allele. We then bred mice having a genotype in which this doubly mutant chromosome was present opposite a fully wild-type chromosome 18. LOH analysis on the DNA of adenomas from *Apc*^{Min}*Dcc*^{-/-}/*Apc*⁺*Dcc*⁺ mice followed by densitometric analysis showed that at least 8/10 of the adenomas in the *cis* compound heterozygous mice had lost the wild-type *Dcc* allele but had retained the mutant (null) *Dcc*⁻ allele (Fig. 2a), as is seen for the linked wild-type *Apc* in virtually all adenomas in the *Min* mice^{32–34}.

Germline heterozygosity for the mutant *Dcc* allele linked to the *Apc*^{Min} allele did not affect polyp initiation or the average size of the adenomas (Table 2). Moreover, histological analysis failed to reveal a change in the morphology of the adenomas (compare Fig. 2b and c). An equal proportion (about 1/20) of the adenomas in *Apc*^{Min}*Dcc*^{+/+}/*Apc*⁺*Dcc*⁺ mice and in *Apc*^{Min}*Dcc*^{-/-}/*Apc*⁺*Dcc*⁺ mice exhibited local invasion, with extension of cyst- and finger-like projections of adenomatous cells deep into muscularis mucosa (compare Fig. 2d and e). Thus, loss of the wild-type allele of the *Dcc* gene occurred in the large majority of intestinal adenomas in the *cis* compound heterozygous mice but it could not be correlated with tumour progression (Fig. 2).

Gastrointestinal epithelial proliferation

To determine whether DCC has a role in regulating the proliferation and differentiation of the intestinal epithelium, we compared the intestinal tissue from five inbred 129/Sv newborn (P1) *Dcc*^{-/-} mice with that from three *Dcc*^{+/+} and five *Dcc*^{+/-} littermates. Inspection of haematoxylin and eosin-stained sections prepared along the length of the duodenal–ileal axis revealed no differences in villus height or cellular architecture (compare Fig. 3a and b). S-phase cells were confined to the intervillus epithelium in all animals, and similar numbers and distribution of BrdU-positive cells within the intervillus epithelium were observed in all animals (compare Fig. 3c and d). Expression of the CCAAT enhancer-binding protein (C/EBPα) provides a sensitive index of the proliferative state of enterocytes, the principal intestinal epithelial cell lineage³⁵. C/EBPα was present at highest concentrations in epithelial cells located in the intervillus epithelium and the lower half of duodenal and jejunal villi in a pattern that was identical in the epithelia of all three sets of mice (Fig. 3f). Thus, loss of *Dcc* function does not affect intestinal epithelial proliferation on the first day after birth.

Epithelial differentiation was also examined using a panel of 9 lectins and 11 antisera. Glycoconjugate production, which can be monitored with lectins, is a sensitive reporter of perturbations in intestinal epithelial differentiation³⁶. Loss of *Dcc* produced no apparent changes in the pattern of lectin binding to the enterocytic

Table 1 Tumours in *Dcc*^{+/-} mice

Genotype*	<i>Dcc</i> ^{+/-} (n = 98)	<i>Dcc</i> ^{+/+} (n = 36)
Epithelial		
Gastrointestinal	9 (9%)†	6 (17%)†
Liver, lung and uterine	11 (11%)	7 (19%)
Mesenchymal		
Leukaemia and lymphoma	11 (11%)	4 (11%)
Sarcoma	8 (8%)	2 (6%)

* Mice are 18–24-month-old F₁ and F₂ 129Sv/B6; n is the number of mice autopsied; † number and fraction (inside the parentheses) of mice that had a given class of tumour or neoplasia. Gastrointestinal lesions are adenomas and were detected by examination of the gastrointestinal lumen under a dissecting microscope. A total of 22 adenomas were found in 9 mice from the *Dcc*^{+/-} cohort (1 mouse with 7 adenomas, 2 with 4 adenomas, 1 with 2 adenomas, 5 with 1 adenoma and 89 mice with no adenomas). Examination of 36 *Dcc*^{+/-} mice revealed adenomas in 6 mice, 1 in each. The majority of the lesions in liver (hepatomas or hepatocarcinomas), lung (adenomas or adenocarcinomas), uterus (adenomas), the haematological lesions and sarcomas were detected only by histological and microscopic examination of the tissues removed at autopsy.

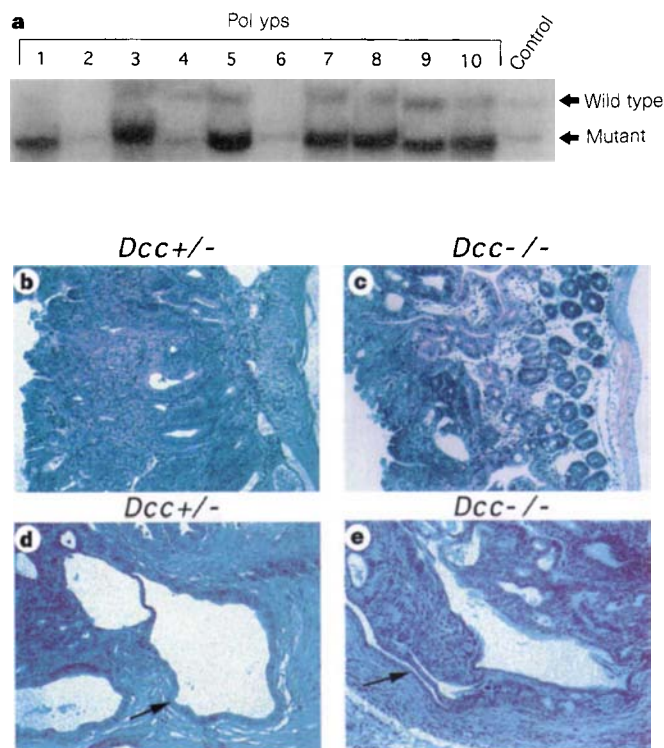


Figure 2 Effect of loss of both alleles of *Dcc* on tumour progression in the mouse intestine. **a**, Southern blot analysis showing the loss of *Dcc*⁺ allele in adenomas extracted from the intestines of compound heterozygote (*Apc*^{Min}*Dcc*^{-/-}/*Apc*⁺*Dcc*⁺) mice. Representative histological sections of two such adenomas from *Apc*^{Min}*Dcc*^{-/-}/*Apc*⁺*Dcc*⁺ mice are shown in panels **c** and **e**. Control is DNA from normal *Dcc*^{+/-} tissues; the band corresponding to the wild-type allele transfers less efficiently in Southern blots because of its larger size (>20 kb). To determine if the *Dcc*⁺ alleles were lost in the adenomas, the intensities of the mutant and the wild-type bands were determined using densitometric measurements and normalized to the intensity of the corresponding band from the control *Dcc*^{+/-} DNA. **b**, A benign adenomatous polyp from a 6-month-old *Apc*^{Min}*Dcc*^{-/-}/*Apc*⁺*Dcc*⁺ mouse stained, as in subsequent panels, with haematoxylin and eosin. **c**, A benign adenomatous polyp from a 6-month-old *Apc*^{Min}*Dcc*^{+/+}/*Apc*⁺*Dcc*⁺ mouse. **d**, An invasive adenocarcinoma from a 6-month-old 129*Apc*^{Min}*Dcc*^{-/-}/*Apc*⁺*Dcc*⁺ mouse. Arrow points to carcinomatous cells that have invaded the muscularis mucosa. **e**, An adenocarcinoma from a 6-month-old 129*Apc*^{Min}*Dcc*^{+/+}/*Apc*⁺*Dcc*⁺ mouse. Arrow points to the site of invasion in the muscularis mucosa by carcinomatous cells. Specimens in panels **b** and **c** are 0.35 mm × 0.48 mm and in panels **d** and **e** are 24 μm × 18 μm.

lineage (Fig. 3c and e). Substance P- and serotonin-producing enteroendocrine subpopulations are good markers of the spatial complexity of this lineage's differentiation programme along the crypt-villus and duodenal-ileal axes³⁷. Loss of *Dcc* did not have any detectable effects on either of these subpopulations (Fig. 3g).

Previous studies have suggested that DCC is most prominently represented in goblet cells³⁸, but loss of *Dcc* did not cause any spatial or developmental changes in goblet cell differentiation, as assessed by the pattern of lectin binding³⁶ (Fig. 3c, e, h). Periodic acid Schiff (PAS) and Alcian blue together stain essentially all goblet cells in the P1 mouse intestine. There was no statistically significant difference ($P > 0.05$) in the number of PAS/Alcian blue-positive goblet cells in the proximal, mid or distal small intestine or in the colon of *Dcc*^{-/-} mice compared to their *Dcc*^{+/-} or *Dcc*^{+/+} littermates.

The Paneth cell lineage is confined to the base of small intestinal crypts and undergoes a characteristic developmental stage-specific sequence of expression of marker gene products from embryonic day 15 (E15) to postnatal day 28 (P28)³⁹. Loss of *Dcc* did not produce any precocious induction of these markers at P1 (data not shown). The basement membrane underlying the intestinal epithelium receives contributions from both epithelial and mesenchymal cell populations⁴⁰. Laminin and collagen IV are prominent components of this basement membrane. Loss of *Dcc* did not affect the amount or distribution of these proteins along the nascent crypt-villus or duodenal-colonic axis (Fig. 3h). Thus, these studies failed to detect

any change in the development of the intestinal epithelium of newborn mice as a result of loss of *Dcc*.

Finally, as with the small intestine and the colon, loss of DCC did not affect histochemical stainings (haematoxylin and eosin, Alcian blue, and PAS), *in situ* binding to lectins or incorporation of BrdU into S-phase cells in the epithelial cells of the stomach (data not shown).

DCC loss and cell migration in intestine

To assess the function of *Dcc* in the adult intestine, we generated chimaeras composed partly of wild-type B6 cells and partly of 129/Sv *Dcc*^{-/-} cells. In the intestine of a B6(-)129/Sv chimaeric mouse, the crypts are known to be populated by either B6 or 129/Sv cells but each villus is populated by cells originating in several adjacent crypts⁴¹. These can be readily distinguished because 129/Sv but not B6 enterocytes bind the lectin *Ulex europaeus* agglutinin type 1 (UEA-1)^{42,43} (Fig. 4).

Some of the B6 *Dcc*^{+/-}(-)129/Sv *Dcc*^{-/-} chimaeras appeared normal at birth and lived to adulthood. In these mice, some of the villi encountered at the border of epithelium derived from ES cells and host blastocyst were polyclonal, being composed of cells originating from both 129/Sv-*Dcc*^{-/-} and B6-*Dcc*^{+/-} crypts. These polyclonal villi contained coherent vertical columns of wholly UEA-1-positive 129/Sv (*Dcc*^{-/-}) enterocytes and adjacent columns of wholly UEA-1-negative B6 (*Dcc*^{+/-}) enterocytes (Fig. 4).

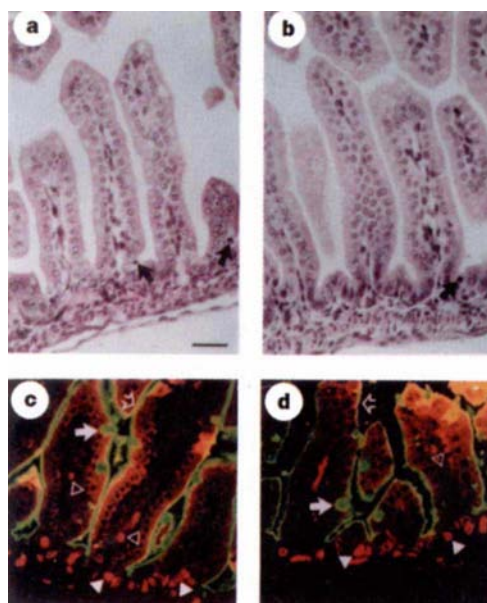
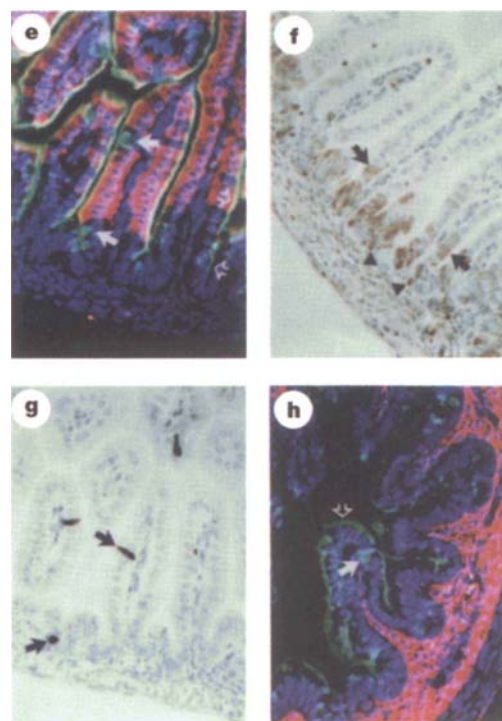


Figure 3 Multilabel immunocytochemical studies of the intestinal epithelium of P1 *Dcc*^{-/-} mice. **a, b**, 129/Sv *Dcc*^{-/-} and *Dcc*^{+/+} mouse jejunum (**a** and **b**, respectively), were stained with haematoxylin and eosin. Arrows point to M-phase (mitotic) cells in the intervillus epithelium. **c**, Jejunum from a P1 *Dcc*^{-/-} mouse. Closed arrowheads point to proliferating, BrdU⁺ (red) cells in the intervillus epithelium. Rare BrdU⁺ lymphocytes are evident in the lamina propria (open arrowheads). Lectin *Dolichos biflorus* agglutinin recognizes a glycoconjugate present on the enterocyte brush border (open arrow) and in the mucus globules of goblet cells (closed arrow). **d**, Comparable segment of 129/Sv *Dcc*^{-/-} jejunum stained as in **c, e**. Triple label of a jejunal segment from a 129/Sv *Dcc*^{-/-} mouse. The section was incubated with rabbit anti-liver fatty-acid binding protein (L-FABP) sera (visualized with Cy3-donkey anti-rabbit Ig), FITC-conjugated cholera toxin B subunit (CTB), and bis-benzimide (dark blue nuclear stain). L-FABP (red-purple) is



present in villus enterocytes. CTB binds to goblet cells (green; closed arrows) and to the brush border of enterocytes located on the villus and in nascent crypts (open arrow). **f**, Duodenum from a *Dcc*^{-/-} mouse. Nuclei were counterstained with haematoxylin. CEBPa-positive enterocytes are present on the lower half of the villi (filled arrows) and in nascent crypts (filled arrowheads). **g**, Jejunum from a *Dcc*^{-/-} mouse stained with rabbit anti-serotonin sera (visualized with IGSS). Nuclei were counterstained with haematoxylin. Immunoreactive enteroendocrine cells (black, arrows) are present in the nascent crypt and in the villus epithelium. **h**, Triple labelling of a section of ileum from a *Dcc*^{-/-} mouse. Collagen IV in the extracellular matrix (red), FITC-conjugated *Helix pomatia* agglutinin (HPA) labelled glycogonjugates located in the enterocytic brush border (green, open arrow) and in the mucus globules of goblet cells (green, filled arrow). Nuclei were visualized with bis-benzimide. Scale bar (in **a**), 25 µm.

Table 2 Effect of inactivation of both alleles of *Dcc* on polyp number and size

Genotype*	Average number of adenomas per mouse	Average size (mm)
<i>Apc^{Min}Dcc^{-/-}/Apc⁺Dcc⁺</i> (<i>n</i> = 47)†	56	2.66 (<i>n</i> = 452)‡
<i>Apc^{Min}Dcc⁺/Apc⁻Dcc⁺</i> (<i>n</i> = 12)†	59	2.69 (<i>n</i> = 335)‡

* Mice are first-, second-, or third-generation backcross of 129Sv/B6 F₁ into 129/Sv; age 6–12 months. † *n* is the number of mice analysed. ‡ *n* is the number of adenomas analysed in mice of the same age (6 months old).

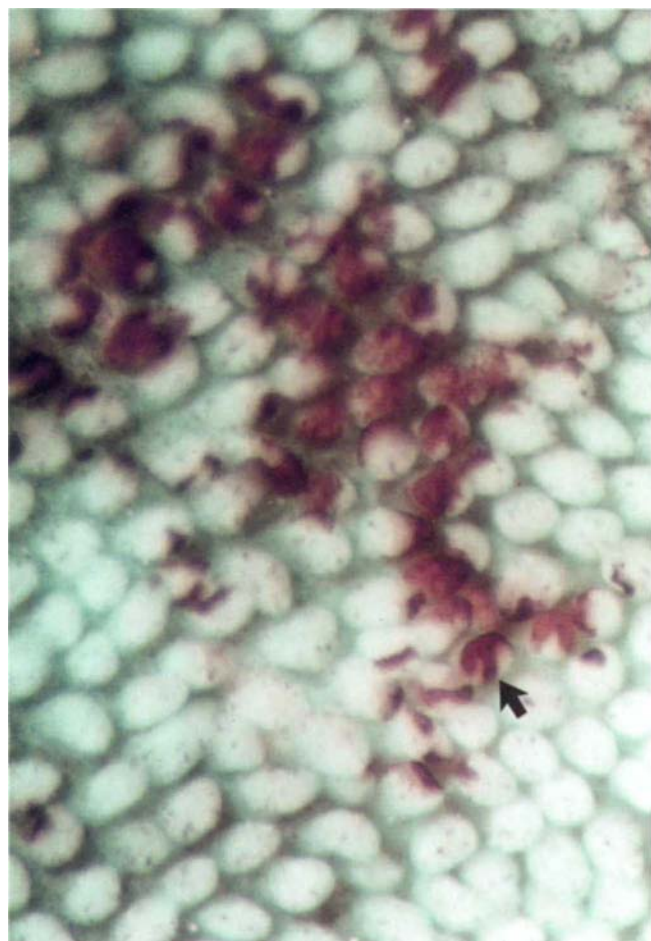


Figure 4 Whole mount of an adult chimaeric B6 (*Dcc^{+/+}*) × 129/Sv (*Dcc^{-/-}*) small intestine. A segment of jejunum from the whole mount preparation is shown, viewed from the luminal surface. The arrow points to a representative polyclonal villus in which three columns of UEA-1-positive (brown) *Dcc^{-/-}* enterocytes extend from adjacent crypts to the tip of the villus and are separated by columns of UEA-1-negative *Dcc^{+/+}* enterocytes (white) supplied by B6 crypts.

The presence of these two easily distinguished cell populations on a single villus provided the opportunity to assess the effects of *Dcc* loss on morphogenesis and cell migration in adult intestinal villi. The ES-derived *Dcc^{-/-}* UEA-1⁺ cellular columns extended from the base to the tip of each polyclonal villus. The border between ES-derived *DCC^{-/-}* and B6 *Dcc^{+/+}* columns was well defined (Fig. 4), indicating that the absence of *Dcc* function did not affect the normal orderly migration of cells from the crypt to the villus tip. Moreover, the two sides of such polyclonal villi were indistinguishable morphologically. Villi that were composed exclusively of UEA-1⁻ *Dcc^{+/+}* B6 cells had the same height as adjacent villi composed of wholly UEA-1⁺ *Dcc^{-/-}* 129/Sv cells (data not shown), indicating that the absence of *Dcc* did not perturb the rate of cell production in crypts and/or the rate of cell loss from villi. Finally, no intestinal or gastric adenomas or tumours of other tissues were found in a cohort of 10 2-year-old chimaeric mice at the time of their death. In these chimaeric mice, which sired only *Dcc^{+/+}* progeny when mated with wild-type mice, the 129/Sv *Dcc^{-/-}* cells contributed to at least 90% of the coat colour.

Hence, by all experimental measures tested, the absence of DCC function did not affect either the normal or the neoplastic mouse gastrointestinal tissue.

Defects in spinal commissural axon

Based on *in vitro* experiments, DCC has been proposed to function as a receptor that mediates the guidance of commissural axons by netrin-1 to the floor plate of the spinal cord¹². To assess the trajectories of commissural axons at E9.5–E11.5 in *Dcc^{-/-}* mice, we labelled these axons in transverse sections of spinal cord at forelimb levels (8 $-/-$, 6 $+/-$ and 5 $+/+$ embryos) using an antibody to TAG-1, a surface protein expressed by commissural axons as they project to the floor plate⁴⁴. Within the dorsal spinal cord of E9.5–E11.5 embryos homozygous for the null *Dcc* allele, we noted a reduction in the number of commissural axons, although those that did extend appeared to adopt a normal dorsal-to-ventral trajectory (Fig. 5 and data not shown). In certain sections (see Fig. 5b and d), some axons were observed that projected all the way to the floor plate. In other sections, however, no axons were seen reaching the floor plate (data not shown). This heterogeneity along the rostro-caudal axis was evident in sagittal views of the spinal cord (Fig. 6; *n* = 10 $-/-$, 6 $+/-$ and 5 $+/+$ embryos). In addition, there appeared to be a misrouting of many of the axons that did project into the ventral spinal cord, with some projecting more medially and others more laterally (Fig. 5d). This impression was confirmed by examining the trajectory of commissural axons using the fluorescent lipophilic dye-DiI injected into the cell bodies of these neurons in the dorsal spinal cord, which diffused down their axons. The pattern of axonal projections visualized in this way at E11.5 was very similar to that observed by TAG-1 immunoreactivity in wild-type embryos (compare Fig. 5a, c and Fig. 7a, b), in heterozygous embryos (Fig. 7c and data not shown), and in 9/9

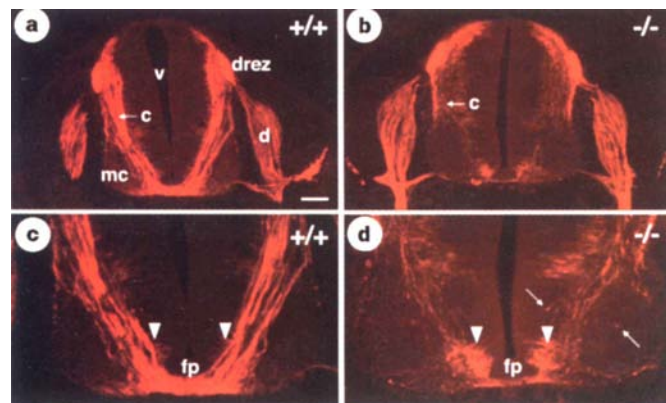


Figure 5 Defects in commissural axon projections in *Dcc^{-/-}* embryos. Trajectories of commissural axons are visualized using an antibody to TAG-1 in sections of wild-type (**a**, **c**) and *Dcc^{-/-}* (**b**, **d**) E11.5 embryos. In *Dcc^{-/-}* embryos, TAG-1⁺ commissural neurons are present but few axons extend into the ventral spinal cord (**b**) and those that do project along aberrant trajectories (arrows in **d**). Projections of sensory axons and motor axons in the ventral roots appear largely normal (**b**). Arrowheads in **c** and **d** indicate a population of TAG-1⁺ cells adjacent to the floor plate. Additional abbreviations: d, dorsal root ganglion; drez, dorsal root entry zone; mc, motor column; v, ventricle; c, commissural axons; fp, floor plate. Scale bars: 100 μ m in **a**, **b**, 50 μ m in **c**, **d**.

Dcc^{-/-} embryos (compare Fig. 5b, d with Fig. 7d–f), and confirmed the misrouting of commissural axons in the ventral spinal cords of *Dcc*^{-/-} embryos. The defects in axonal projections observed in and around the spinal cord appeared specific to commissural axons, inasmuch as the trajectories of sensory and motor axons (the latter of which express DCC¹²) appeared normal, as visualized using the TAG-1 marker (compare Fig. 5a and b) and using an antibody to neurofilament protein (data not shown). Thus, *Dcc*^{-/-} embryos show defects in commissural axon projections that are similar to those seen in *netrin-1*-deficient mice¹⁷ but are more severe (see Conclusion).

Defects in brain development in *Dcc*^{-/-} mice

In addition to defects in spinal cord development, *netrin-1*-deficient animals show multiple defects in brain development¹⁷. Similar defects were observed in the brains of *Dcc*^{-/-} mice. The corpus callosum and hippocampal commissure appeared to be completely absent (compare Fig. 8a and b). The axons that normally form these commissures appeared to be present but failed to cross the midline

and remained ipsilateral, projecting to aberrant locations and forming tangles ('Probst bundles'). The anterior commissure in *Dcc*^{-/-} mice was also severely reduced: the large anterior and posterior limbs of the anterior commissure (Fig. 8c) did not form and join near the midline; Fig. 8d shows the largest remnant of this commissure that was detected in *Dcc*^{-/-} mice. As in *netrin-1*-deficient animals, these defects did not reflect a generalized defect in commissure formation, as the habenular and posterior commissures both appeared to be intact (Fig. 8e, f). In the brainstem of *Dcc*^{-/-} mice, a commissure that is not normally present was observed in the junctional region between hindbrain and midbrain (compare Fig. 8g and h). In addition, the pontine nuclei at the base of the rostral hindbrain appeared to be absent in the mutant animals (compare Fig. 8i and j). Thus, the defects seen in the brain of *Dcc*^{-/-} animals were similar to those observed in *netrin-1*-deficient animals¹⁷.

Conclusion

Is DCC a tumour-suppressor gene? The experiments described here were initially designed to assess the role of the human DCC

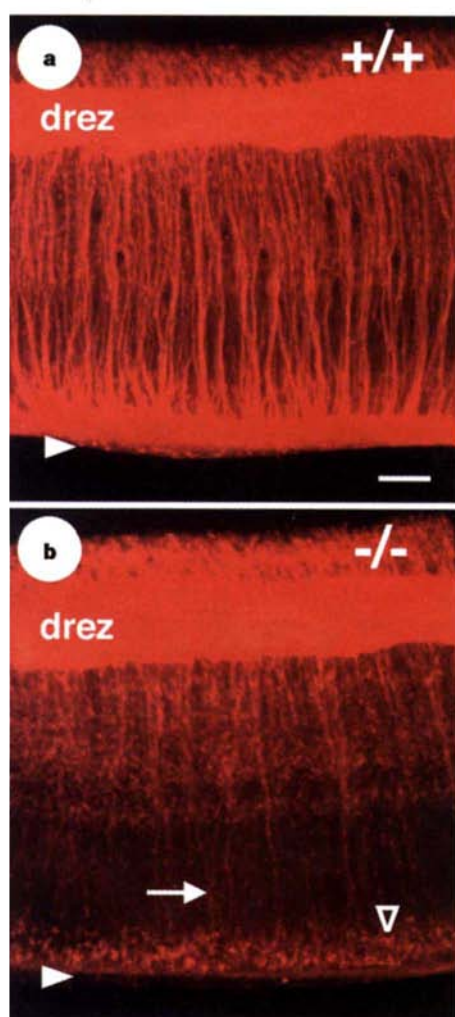


Figure 6 Few commissural axons reach the floor plate in *Dcc*^{-/-} embryos. Sagittal views (side views) of spinal cords from a wild-type (a) and a *Dcc*^{-/-} (b) E11.5 embryo showing commissural axons visualized by TAG-1 immunohistochemistry. Dorsal is up, ventral is down (arrowhead indicates floor plate) (drez indicates sensory axons in the dorsal root entry zone). The characteristic microsegmented projections of commissural axons along a dorso-ventral trajectory to the floor plate are visible in the wild-type embryo (a). In the mutant (b) many fewer axons extend within the dorsal spinal cord, and only a few reach the floor plate (arrow). Outline arrowhead indicates TAG-1⁺ cells adjacent to floor plate. Scale bar, 100 μm.

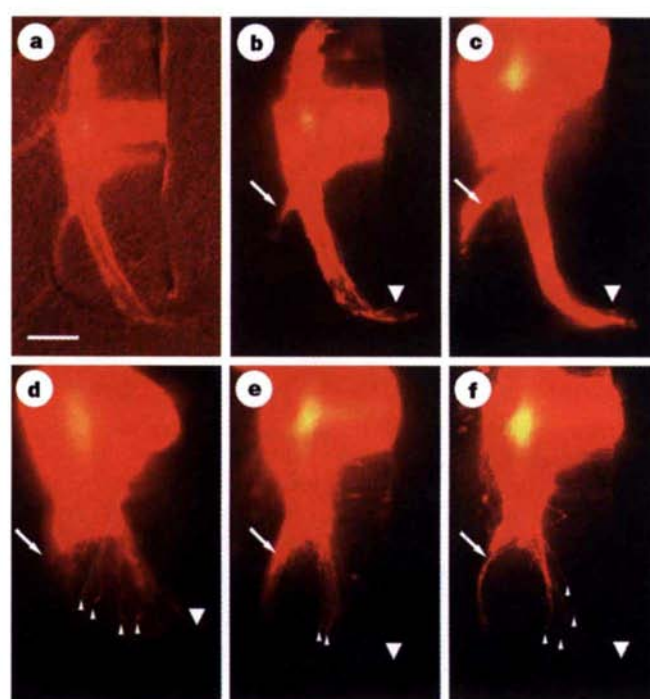


Figure 7 Defects in commissural axon projections revealed by dye-tracing. Dil was injected into the dorsal spinal cord of wild-type (a, b), heterozygous (c) and homozygous mutant *Dcc*^{-/-} E11.5 embryos (d–f) and allowed to diffuse down commissural axons. Axonal projections were visualized in transverse vibratome sections. In b–f, large arrowheads indicate floor plate and arrows indicate axons projecting along the lateral edge of the motor column (presumptive ipsilaterally projecting axons). Note that less Dil was injected in the wild-type embryo (a, b) than in the other embryos (c–f). a, b, Combined differential interference contrast and fluorescence micrograph (a), and fluorescence micrograph (b), of a wild-type embryo, showing the normal trajectory of commissural axons to the floor plate. Note that ipsilaterally projecting axons originating in the dorsal spinal cord do not express TAG-1 (compare to Fig. 5a and c). c, A similar pattern of projection is observed in *Dcc*^{-/-} embryos (*n* = 12/12). d–f, Trajectory of axons labelled from the dorsal spinal cord in 3 different *Dcc*^{-/-} embryos. The trajectory of presumptive commissural axons revealed by dye-tracing in *Dcc*^{-/-} embryos is similar to that seen by TAG-1 labelling (compare to Fig. 5b and d): many fewer axons project into the ventral spinal cord; some axons project ventromedially but are not particularly directed towards the floor plate (e, f), and many axons wander within the motor column (d). Small vertical arrowheads in d–f indicate a sampling of commissural growth cones. Few axons reach the floor plate (large arrowheads in d–f). Similar results were observed in 9/9 *Dcc*^{-/-} embryos.

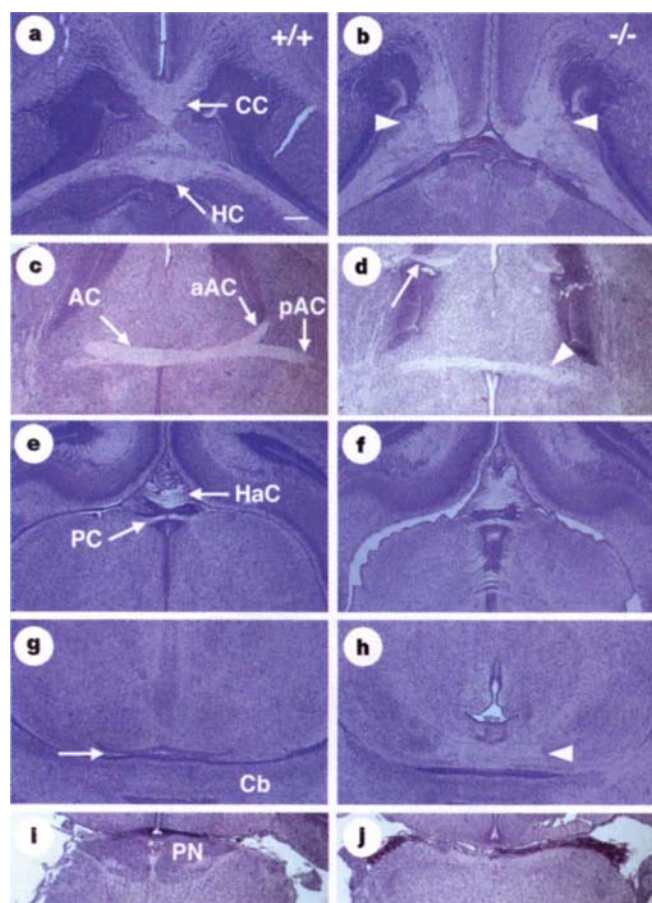


Figure 8 Defects in brain commissures and absence of pontine nuclei in *Dcc*^{-/-} embryos. Horizontal sections from brains of wild-type or heterozygous neonatal pups (**a, c, e, g, i**), and homozygous mutant pups (**b, d, f, h, j**) stained with haematoxylin and eosin (rostral is up in each). The phenotype of wild-type and heterozygous pups were indistinguishable. **a, b**, Corpus callosum (CC) and hippocampal commissure (HC) of a wild-type (**a**) and *Dcc*^{-/-} (**b**) pup (arrowheads, Probst bundles). **c, d**, The anterior commissure (AC) in a wild-type (**c**) and a homozygous *Dcc*^{-/-} mutant (**d**) pup (aAC, anterior limb; pAC, posterior limb). In homozygous mutant brains an aberrant bundle of axons (arrow in **d**) was observed approaching the midline at more rostral levels, which may consist of misrouted axons that normally form the anterior limb. The posterior limbs were much reduced but not, apparently, completely absent, and in most mutants a reduced commissure was observed (arrowhead). **e, f**, The habenular commissure (HaC) and posterior commissure (PC) are present in wild-type (data not shown), heterozygous (**e**), and homozygous mutant (**f**) pups. **g, h**, In *Dcc*^{-/-} pups (**h**), a thick commissure (arrowhead) is observed in the roof of the fourth ventricle in the region of the hindbrain-midbrain junction, that is not observed in wild-type pups (arrow in **g**) or heterozygous pups (data not shown); Cb, cerebellum. **i, j**, The pontine nuclei (PN) at the base of the rostral hindbrain in wild-type (data not shown) and heterozygous (**i**) pups are not detected in *Dcc*^{-/-} pups (**j**). Scale bars: 175 μ m in **a, b, e, f, i, j**; 135 μ m in **c, d**; 150 μ m in **g, h**; 200 μ m in **i, j**.

gene in colonic tumorigenesis through manipulation of its mouse homologue. Although we observed a small number of tumours in several tissues in aged *Dcc*^{+/-} mice, this number was no greater than that observed in wild-type mice (Table 1), and those lesions examined genetically gave no evidence of loss of the remaining wild-type *Dcc* allele. A potentially more sensitive test of a tumour-suppressor role of *DCC* involved inactivation of both alleles of *Dcc* in mouse intestinal adenomas. Because *DCC* has been described as a tumour-suppressor gene whose expression is eliminated during colonic tumour progression following *APC* inactivation^{3,4,25,26}, we expected that its loss would accelerate the progression of, or modify the phenotype of polyps initiated in the *Apc*^{Min/+} mice. We observed no such effect (Table 2 and Fig. 2). A third line of investigation addressed any part played by the *Dcc* gene in morphogenesis of colonic crypts and villi, and proliferation and development of the associated epithelial cell populations in the mouse. The absence of *Dcc* had no effect on proliferation or differentiation of intestinal epithelial cells or intestinal morphogenesis (Figs 3 and 4). Taken together, these three lines of investigation argue that *Dcc* plays little if any part in the physiology of the intestinal epithelium in the mouse. It should also be emphasized, however, that although these results weaken the candidacy of the *DCC* gene as an important regulator of intestinal epithelial cell proliferation in the mouse, they do not rule out definitively its role in human colon cancer pathogenesis.

The strongest evidence in support of the role of *DCC* as a tumour suppressor stems from observations of the reduction or loss of *DCC* RNA in cell lines or xenografts derived from human colon carcinomas^{1,8,9}. However, a similar LOH of 18q and loss of *DCC* RNA expression has been observed in many pancreatic tumours⁴⁵ in which another gene on chromosome 18q21, termed *DPC4*, has recently been identified as the tumour-suppressor gene that is the

target of genetic inactivation⁴⁶. The importance of *DPC4* in pancreatic tumorigenesis is strongly supported by the discovery of a number of inactivating mutations affecting this gene in a series of tumour samples⁴⁶. Thus, in human colon cancer, as is seemingly the case in pancreatic cancer, the loss of *DCC* expression may be a consequence of events affecting a linked gene. According to this model, the observed LOH of 18q21 affects not only the *DCC* gene but other neighbouring genes as well, one or more of which is the target of inactivation during colon tumour progression. The definitive proof of this possibility can only come from the identification of a gene on 18q that undergoes LOH and whose retained allele is also mutated in most (up to 65% of) colonic tumours. This linked gene is unlikely to be either *DPC4* or *JV18* because their retained alleles appear to be mutated in only a fraction of human colon tumours that suffers 18q LOH⁹.

Requirement of *DCC* for axon projections. Our results indicate profound effects of loss of *DCC* function on the development of axonal projections in the central nervous system. These defects are observed in the same classes of axons that are affected in *netrin-1*-deficient animals¹⁷. Our observations are therefore consistent with the hypothesis that *DCC* is a component of an axonal receptor for netrin-1¹².

Although the overall similarity in phenotypes in the *Dcc*^{-/-} and *netrin-1*-deficient mice is striking, spinal commissural axons appear more foreshortened in the dorsal spinal cord in *Dcc*^{-/-} mice (compare this study to ref. 17). This difference could be due to the possibility that the studied *netrin-1* allele was not a complete null allele, and that some residual netrin-1 function was present in those animals¹⁷. An alternative possibility is that *DCC* is required not only to mediate responses to netrin-1 but also to mediate the responses of commissural axons to other cues that collaborate with netrin-1 to guide these axons.

We also note that some commissural axons do in fact succeed in reaching the floor plate in the *Dcc*^{-/-} mice (Figs 5 and 6). These observations point to the existence of a DCC-independent mechanism for guidance of commissural axons to the floor plate. It is possible that netrin-1 itself can function through a DCC-independent mechanism to guide commissural axons in the *Dcc*^{-/-} mice. Alternatively, cues distinct from netrin-1 might be present that guide commissural axons toward the floor plate (see ref. 17) and function in a DCC-independent mechanism.

DCC and netrin-1 in brain. The finding of defects in brain morphogenesis in *Dcc*^{-/-} animals that are similar to those observed in *netrin-1*-deficient animals suggests that DCC is in the response pathway for netrin-1 effects in the brain. It is not known how the defects in the brain arise in either the *netrin-1*-deficient or the *Dcc*^{-/-} animals, although the distribution of *netrin-1* messenger RNA has suggested that netrin-1 might function as a guidance cue for the cells and axons that are affected¹⁷. This is true not just for the defects in axonal projections that are observed, but also for the pontine nuclei, the absence of which might reflect a failure of migration of postmitotic pontine neurons (discussed in ref. 17). If so, the absence of pontine nuclei would indicate that DCC functions to guide migrating cells in addition to axons.

Although the precise role of netrin-1 and DCC in guiding migrations in the brain remains to be elucidated, our finding of similar defects in *Dcc*^{-/-} and *netrin-1*-deficient animals parallels the observation, in *C. elegans* and *Drosophila*, that mutations in homologues of *Dcc* and *netrin-1* give similar neural phenotypes^{22,23} and provides strong support for the hypothesis that the wiring of the nervous system involves rules and mechanisms that are highly conserved among nematodes, insects and vertebrates⁴⁷. □

Methods

Generation and analysis of mice with *Dcc*^{X3} mutation. The *Dcc*^{X3} targeting vector was constructed using a fragment of the *Dcc* gene that extends from intron two to intron three. This DNA fragment was isolated from a 129/SV mouse genomic library using a probe containing human exon 3 (gift of B. Vogelstein) which encodes most of the protein's second immunoglobulin-like domain. Sequencing of the isolated fragment identified a stretch of 283 nucleotides that was 100% identical to the reported sequence of exon 3 (codons 138–232) of *Mus musculus Dcc* gene. EST data bank searches also revealed that the predicted amino-acid sequence encoded by the isolated exon is 41% and 40% identical to a stretch of amino acids in rat and human neogenin, respectively, and 93% identical to codons 138–232 in human DCC. Embryonic stem cell culture, generation of chimaeric mice, Southern and western blot analyses were performed according to standard procedures. C57BL/6 *Apc*^{Min/+} (Jackson Laboratory, Bar Harbor, Maine) and 129/Sv *Dcc*^{+/-} animals were crossed to generate 129Sv/B6 F1 *Apc*^{Min/+}*Dcc*^{+/-} mice. These F₁ mice were then backcrossed to 129/Sv mice; the compound heterozygote progeny of this latter cross had the mutant allele of both the *Apc* and the *Dcc* gene in the *cis* configuration. Homozygous mutant *Dcc*^{-/-} ES cells were generated from 129/Sv *Dcc*^{+/-} ES cells using a previously described method⁴⁸.

Immunohistochemical analysis of intestines. Single and multilabel immunohistochemical analyses of postnatal day 1 (P1) 129/Sv *Dcc*^{-/-}, *Dcc*^{+/-} and *Dcc*^{+/+} mice were conducted in a single blinded fashion and as described previously^{42,43}. A panel of 11 antisera were used: (1) goat anti-BrdU (S-phase cells); (2) rabbit anti-rat liver fatty-acid binding protein (villus enterocytes); (3) rabbit anti-intestinal fatty-acid binding protein (enterocytes); (4) rabbit anti-C/EBPα (proliferating epithelial cells); (5) rabbit anti-pan-cadherin (epithelial cadherins); (6) rabbit anti-laminin; (7) rabbit anti-collagen IV; (8) rabbit anti-serotonin; (9) rabbit anti-substance P; (10) rabbit anti-cryptdin (Paneth cells); and (11) rabbit anti-enhancing factor (Paneth cells). Nine lectins were used to examine differentiation of intestinal epithelial cells: (1) Jacalin (*Artocarpus integrifolia* agglutinin); (2) peanut agglutinin; (3) *Tirchosantes kirilowii*; (4) *Dolichos biflorus* agglutinin; (5) *Helix pomatia* agglutinin; (6) *Griffonia simplicifolia*; (7) *Ulex europaeus* agglutinin type I; (8) cholera toxin B subunit; and (9) *Sanbucus nigra* agglutinin. The number of goblet cells was defined in the proximal, mid and distal thirds of the small intestine and in the

colon. PAS/Alcian blue-stained goblet cells in 20 well-oriented, nascent crypt-villus units were counted per region per mouse (*n* = 2 animals per genotype). Paired Student's *t*-test was performed to assess whether any statistically significant differences existed in the total number of goblet cells per region of *Dcc*^{-/-}, *Dcc*^{+/-} and *Dcc*^{+/+} intestines.

Spinal cord histochemistry and brain histology. Immunohistochemistry on transverse sections of spinal cord was performed with antibodies to TAG-1 (4D7, gift of M. Yamamoto) and NF-M (gift of V. Lee) as previously described¹⁷ except that 20-μm-thick sections were used and 0.2% fish skin gelatin was added to PTH buffer. Whole-mount immunohistochemistry was performed essentially as described previously¹⁷ (protocol available on request). Dye-tracing experiments were performed as described previously¹⁹.

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1. Fearon, E. R. *et al.* Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* **247**, 49–56 (1990).
2. Cavenee, W. K. *et al.* Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature* **305**, 779–784 (1983).
3. Boland, C. R., Sato, J., Appelman, H. D., Bresalier, R. S. & Feinberg, A. P. Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. *Nature Med.* **1**, 902–909 (1995).
4. Vogelstein, B. *et al.* Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.* **319**, 525–532 (1988).
5. Tanaka, K. *et al.* Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 5 or 18. *Nature* **349**, 340–342 (1991).
6. Goyette, M. C. *et al.* Progression of colorectal cancer is associated with multiple tumor suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. *Mol. Cell. Biol.* **12**, 1387–1395 (1992).
7. Cho, K. R. *et al.* The DCC gene: structural analysis and mutations in colorectal carcinomas. *Genomics* **19**, 525–531 (1994).
8. Kikuchi-Yanoshita, R., Konishi, M., Fukunari, H., Tanaka, K. & Miyaki, M. Loss of expression of the DCC gene during progression of colorectal carcinomas in familial adenomatous polyposis and non-familial adenomatous polyposis patients. *Cancer Res.* **52**, 3801–3803 (1992).
9. Thiagalingam, S. *et al.* Evaluation of candidate tumor suppressor genes on chromosome 18 in colorectal cancers. *Nature Genet.* **13**, 343–346 (1996).
10. Narayanan, R. *et al.* Antisense RNA to the putative tumor-suppressor gene DCC transforms Rat-1 fibroblasts. *Oncogene* **7**, 553–561 (1992).
11. Klingelhutz, A. J., Hedrick, L., Cho, K. R. & McDougall, J. K. The DCC gene suppresses the malignant phenotype of transformed human epithelial cells. *Oncogene* **10**, 1581–1586 (1995).
12. Keino-Masu, K. *et al.* Deleted in colon cancer (DCC) encodes a netrin receptor. *Cell* **87**, 175–185 (1996).
13. Tessier-Lavigne, M., Placzek, M., Lumsden, A. G. S., Dodd, J. & Jessell, T. M. Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775–778 (1988).
14. Placzek, M., Tessier-Lavigne, M., Jessell, T. M. & Dodd, J. Orientation of commissural axons in vitro in response to a floor plate-derived chemoattractant. *Development* **110**, 19–30 (1990).
15. Serafini, T. *et al.* The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78**, 409–424 (1994).
16. Kennedy, T. E., Serafini, T., de la Torre, J. R. & Tessier-Lavigne, M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425–435 (1994).
17. Serafini, T. *et al.* Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001–1014 (1996).
18. Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G. & Hedgecock, E. M. UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in *C. elegans*. *Neuron* **9**, 873–881 (1992).
19. Wadsworth, W. G., Bhatt, H. & Hedgecock, E. M. Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in *C. elegans*. *Neuron* **16**, 35–46 (1996).
20. Mitchell, K. J. *et al.* Genetic analysis of *Netrin* genes in *Drosophila*: netrins guide CNS commissural axons and peripheral motor axons. *Neuron* **17**, 203–215 (1996).
21. Harris, R., Sabatelli, L. M. & Seeger, M. A. Guidance cues at the *Drosophila* CNS midline: identification and characterization of two *Drosophila* Netrin/UNC-6 homologs. *Neuron* **17**, 217–228 (1996).
22. Chan, S. S.-Y. *et al.* UNC-6, a *C. elegans* homolog of DCC (deleted in colorectal cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell* **87**, 187–195 (1996).
23. Kolodziej, P. A. *et al.* *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell* **87**, 197–204 (1996).
24. Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc. Natl. Acad. Sci. USA* **83**, 9065–9069 (1986).
25. Fearon, E. R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **61**, 759–767 (1990).
26. Fearon, E. in *The Molecular Basis of Cancer* (ed. Mendelsohn, J.) 340–355 (Saunders, Philadelphia, 1995).
27. Moser, A. R., Pitot, H. C. & Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**, 322–324 (1990).
28. Su, L. K. *et al.* Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* **256**, 668–670 (1992).
29. Moser, A. R., Dove, W. F., Roth, K. A. & Gordon, J. I. The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. *J. Cell Biol.* **116**, 1517–1526 (1992).
30. Justice, M. J. *et al.* A molecular genetic linkage map of mouse chromosome 18 reveals extensive linkage conservation with human chromosomes 5 and 18. *Genomics* **13**, 1281–1288 (1992).
31. Luongo, C. *et al.* Mapping of multiple intestinal neoplasia (Min) to proximal chromosome 18 of the mouse. *Genomics* **15**, 3–8 (1993).
32. Luongo, C., Moser, A. R., Gledhill, S. & Dove, W. F. Loss of *Apc* in intestinal adenomas from Min mice. *Cancer Res.* **54**, 5947–5952 (1994).
33. Levy, D. B. *et al.* Inactivation of both APC alleles in human and mouse tumors. *Cancer Res.* **54**, 5953–5458 (1994).
34. Laird, P. W. *et al.* Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* **81**, 197–205 (1995).
35. Chandrasekaran, C. & Grodon, J. I. Cell lineage-specific and differentiation-dependent patterns of CCAAT/enhancer binding protein alpha expression in the gut epithelium of normal and transgenic mice. *Proc. Natl. Acad. Sci. USA* **90**, 8871–8875 (1993).
36. Falk, P., Roth, K. A. & Gordon, J. I. Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *Am. J. Physiol.* **266**, G987–1003 (1994).

37. Roth, K. A. & Gordon, J. I. Spatial differentiation of the intestinal epithelium: analysis of enteroendocrine cells containing immunoreactive serotonin, secretin, and substance P in normal and transgenic mice. *Proc. Natl Acad. Sci. USA* **87**, 6408–6412 (1990).
38. Hedrick, L. et al. The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev.* **8**, 1174–1183 (1994).
39. Bry, L. et al. Paneth cell differentiation in the developing intestine of normal and transgenic mice. *Proc. Natl Acad. Sci. USA* **91**, 10335–10339 (1994).
40. Louvard, D., Kedinger, M. & Hauri, H. P. The differentiating intestinal epithelial cell: establishment and maintenance of functions through interactions between cellular structures. *Annu. Rev. Cell Biol.* **8**, 157–195 (1992).
41. Schmidt, G. H., Winton, D. J. & Ponder, B. A. Development of the pattern of cell renewal in the crypt-villus unit of chimaeric mouse small intestine. *Development* **103**, 785–790 (1988).
42. Hermiston, M. L., Green, R. P. & Gordon, J. I. Chimeric-transgenic mice represent a powerful tool for studying how the proliferation and differentiation programs of intestinal epithelial cell lineages are regulated. *Proc. Natl Acad. Sci. USA* **90**, 8866–8870 (1993).
43. Hermiston, M. L. & Gordon, J. I. In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. *J. Cell Biol.* **129**, 489–506 (1995).
44. Dodd, J., Morton, S. B., Karageorgos, D., Yamamoto, M. & Jessell, T. M. Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron* **1**, 105–116 (1988).
45. Hohne, M. W., Halatsch, M. E., Kahl, G. F. & Weinle, R. J. Frequent loss of expression of the potential tumor suppressor gene DCC in ductal pancreatic adenocarcinoma. *Cancer Res.* **52**, 2616–2619 (1992).
46. Hahn, S. A. et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**, 350–353 (1996).
47. Goodman, C. S. The likeness of being: phylogenetically conserved molecular mechanisms of growth cone guidance. *Cell* **78**, 353–356 (1994).
48. Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A. & Seidman, J. G. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* **12**, 2391–2395 (1992).
49. Stoeckli, E. T. & Landmesser, L. T. Axonin-1, Nr-CAM, and Ng-CAM play different roles in the in vivo guidance of chick commissural neurons. *Neuron* **14**, 1165–1179 (1995).
50. Ekstrand, B. C., Mansfield, T. A., Bigner, S. H. & Fearon, E. R. *Dcc* expression is altered by multiple mechanisms in brain tumors. *Oncogene* **11**, 2393–2402 (1995).

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Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking *Brca2*

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Inherited mutations in the human *BRCA2* gene cause about half of the cases of early-onset breast cancer. The embryonic expression pattern of the mouse *Brca2* gene is now defined and an interaction identified of the *Brca2* protein with the DNA-repair protein Rad51. Developmental arrest in *Brca2*-deficient embryos, their radiation sensitivity, and the association of *Brca2* with Rad51 indicate that *Brca2* may be an essential cofactor in the Rad51-dependent DNA repair of double-strand breaks, thereby explaining the tumour-suppressor function of *Brca2*.

Individuals with mutations in either the *BRCA1* or *BRCA2* genes have a dominant cancer predisposition^{1,2}. Neoplasia appears to be associated with loss of heterozygosity of the non-mutated alleles in tumours that arise in these patients, suggesting that both of these two genes are tumour suppressors. Inherited mutations in *BRCA1* cause breast cancer and ovarian cancer, whereas inherited mutations in *BRCA2* cause mostly breast cancer with a lower risk of ovarian cancer compared to *BRCA1* (refs 1, 3, 4). The *BRCA2* gene encodes a 3,418-amino-acid protein with no significant homology to any known protein^{5,6}. The gene has been extensively screened for mutations in breast tumours, and the results indicate that mutations in *BRCA2* are very rare in non-familial primary breast tumours^{4–10}. The mouse *Brca2* protein consists of 3,328 amino acids and the overall identity between the human and mouse proteins is 58%, although some regions are more highly conserved¹¹.

The mouse *Brca2* gene is expressed in adult thymus, testis and ovary and in the midgestation embryo¹¹. It is also expressed in mammary epithelial cells and appears to be coordinately regulated with *Brca1* expression¹². Both *Brca1* and *Brca2* are highly expressed in rapidly proliferating cells, with the expression peaking at the G1/S

boundary of the cell cycle¹². This expression profile and the common breast cancer phenotype seen in patients with mutations in these genes suggest that *Brca1* and *Brca2* may possibly be acting in the same pathway.

Some insight into *BRCA1* function was provided by the observation of an association between *BRCA1* and HsRad51 (ref. 13). HsRad51 is the human homologue of the *Escherichia coli* protein RecA and of yeast ScRad51, a member of the RAD52 epistasis group in *Saccharomyces cerevisiae*^{14,15}. ScRad51 and its homologues are key components of the double-strand-break repair pathway, as shown by phenotypic and biochemical analysis^{14,16–20}. ScRad51 is also essential for mitotic and meiotic recombination^{14,15,21,22}. Mutation of *rad51* in yeast and mammalian cells results in chromosome loss^{14,17}. HsRad51 forms foci in S-phase nuclei and these foci are also immunoreactive against anti-*BRCA1* antibodies¹³. Moreover, *BRCA1* and HsRad51 colocalize in preparations of meiotic chromosomes. HsRad51 coimmunoprecipitates with *BRCA1*, confirming that these proteins form a complex. The HsRad51–*BRCA1* association suggests that *BRCA1* participates in Rad51 functions.

We have examined the function of *Brca2* in the mouse. *Brca2*-deficient embryos are normal throughout early pre- and postimplantation development, but development arrests at the same time as the onset of detectable *Brca2* expression after day 6.5 of gestation.

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Embryonic expression of *Brca2* is at least in part associated with rapidly proliferating tissues. We have also defined an interaction between a conserved region of *Brca2* and MmRad51 (mouse homologue of Rad51) in a yeast two-hybrid assay. This result was confirmed in mammalian cells and we show that both genes are coexpressed in the embryo. *Brca2*-deficient embryos were found to be hypersensitive to γ -irradiation. This shows that the Rad51/*Brca2* interaction is functionally significant and suggests a mechanism by which *Brca2* might function as a tumour-suppressor gene.

Brca2 targeting and phenotype analysis

We have mutated the *Brca2* locus in mice using embryonic stem cell technology (Fig. 1). A positive/negative selection strategy was applied to disrupt exon 11 by deleting amino acids 626–1,437 (Fig. 1a, c). The targeting vector, pBrca2TV containing the human *Hprt* minigene as the positive-selection marker and the HSV *tk* gene as the negative-selection marker, was electroporated into AB2.2 ES cells (Fig. 1b). Clones carrying the mutant *Brca2* allele, *brca2*^{Brdm1}, were identified by Southern blotting (Fig. 1d). Four of the eight *brca2*^{Brdm1}/+ ES cell lines were injected into blastocysts. Most of the chimaeric mice born transmitted the *brca2*^{Brdm1} allele to their progeny. The heterozygous mice are healthy and fertile. As loss of BRCA2 function in humans results in tumorigenesis, these mice are currently being monitored for development of neoplasia. So far, none of the mice have shown any signs of tumour formation until at least eight months of age.

Six litters from *brca2*^{Brdm1}/+ intercross matings failed to produce any *brca2*^{Brdm1}/*brca2*^{Brdm1} offspring, indicating that *Brca2* was essential for embryonic development. To determine the cause and time of embryonic lethality, embryos at various stages of development were dissected from heterozygous females mated to heterozygous males. At E6.5 of development, 16 embryos were histologically examined. All embryos were normal at this stage. However, by E7.5 about 25% ($n = 45$) of the embryos exhibited a mutant phenotype. At this stage of development, normal embryos have undergone primitive-streak formation²³ (Fig. 2a). Mesoderm is produced and cells start to migrate to form anterior mesoderm around the embryonic ectoderm, and in addition migrate into extraembryonic ectoderm to form structures such as amnion, chorion and allantois. In contrast, the mutant class of embryos resemble E6.5 embryos with very little morphologically visible mesoderm (Fig. 2b, c). However, the differentiation of embryonic ectoderm into mesoderm was confirmed by the presence of *Brachyury* gene expression in the posterior end of the mutant embryo (Fig. 3a, b). The embryonic ectoderm of mutant and wild-type embryos consist of a pseudocolumnar layer of cells, with few dividing cells present. The embryos have a well developed layer of extra-embryonic ectoderm. The amniotic cavity forms and amniotic folds consisting of only the extraembryonic

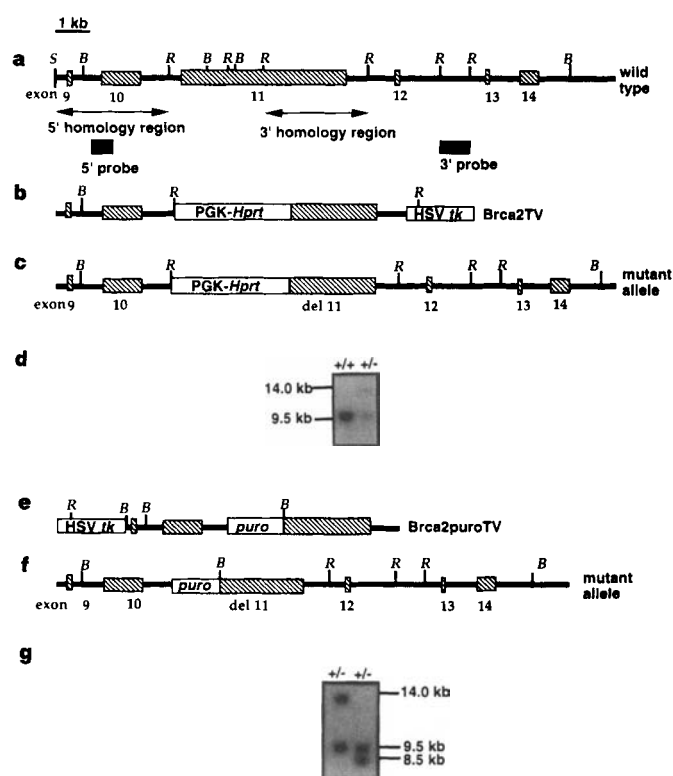


Figure 1 Disruption of *Brca2* gene in ES cells. **a**, Restriction map of the *Brca2* genomic fragment containing exon 9–15 is shown. Restriction sites shown here are *EcoRI* (R) and *BamHI* (B). *SalI* (S) is from the cloning vector. Double-headed arrows correspond to the 5' and 3' homology regions and the dark shaded boxes show the probes used. **b**, Restriction map of the targeting vector pBrca2TV. **c**, Expected restriction map of the mutated *Brca2* locus. A 2.8-kb genomic region is deleted and replaced by the 3.6-kb *Hprt* gene. **d**, Southern analysis to identify heterozygous ES cells by digesting genomic DNA with *BamHI*. The 3' probe detects a 9.5-kb wild-type band and a 14.0-kb mutant band. **e**, Restriction map of the targeting vector pBrca2puroTV electroporated into heterozygous ES cells. A *BamHI* restriction site was introduced between the *puro* gene and 3' homology arm. The HSV *tk* gene was ligated to 5' homology region. **f**, Expected restriction map of the mutant *Brca2* locus. A 2.8-kb genomic region is deleted and replaced by the 1.4-kb *puro* gene. **g**, Southern analysis to identify the second targeting event in heterozygous ES cells by digesting genomic DNA with *BamHI*. The 3' probe detects a 9.5-kb wild-type band and a 14.0-kb mutant band. The second targeting results in a 8.5-kb mutant band.

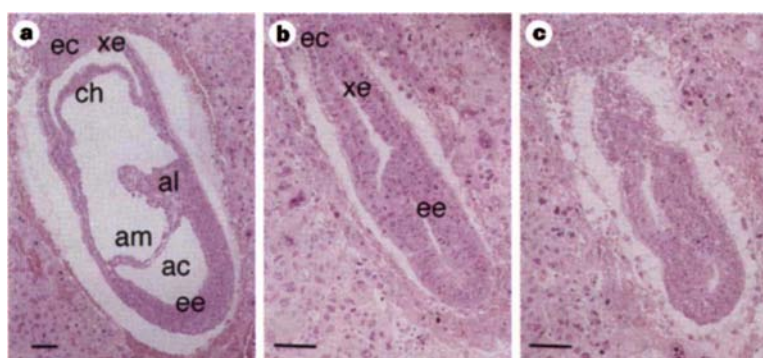


Figure 2 Comparison of *Brca2* mutant embryos with their wild-type littermates. **a**, E7.5 wild-type embryo. **b** and **c**, E7.5 *brca2*^{Brdm1}/*brca2*^{Brdm1} embryos. ac, Amniotic

cavity; al, allantois; am, amnion; ch, chorion; ec, ectoplacental cone; ee, embryonic ectoderm; xe, extraembryonic ectoderm. Scale bars, 100 μ m.

ectoderm layer are evident. In addition to the absence of amnion, mutant embryos completely lack allantois and chorion. However, the visceral and parietal endoderm appear to be normal except for the overall reduction in size.

Brca2 in cell proliferation and survival

The fact that *Brca2* mutant embryos initiate mesoderm formation suggests that the developmental block is not due to lack of differentiation, rather this may result from a proliferation defect. To test this idea, we attempted to generate homozygous mutant ES cells. Given that the developmental block occurred after E6.5 and ES cells are believed to be functionally equivalent to 5.5 day embryonic ectoderm, it should be possible to isolate such cell lines. One of the *brca2^{Brdm1}*/+ES cell clones (D6) was transfected with pBrca2-PuroTV, a targeting vector very similar to the pBrca2TV, except that it had a puromycin gene as the positive-selection marker (Fig. 1e). A total of 12 targeted clones were obtained out of 150 *puro^r* and

FIAU^r cells. However, Southern analysis revealed that none of these 12 clones had disrupted the remaining wild-type *Brca2* allele; instead, all the clones had retargeted the *brca2^{Brdm1}* allele (Fig. 1g). The failure to obtain any homozygous mutant ES cells is highly significant ($P < 0.01$), which suggests that the *Brca2* gene is required for the survival and/or proliferation of ES cells.

To correlate the expression of *Brca2* with the phenotype seen in *Brca2*-deficient embryos, we examined its expression in embryos using *in situ* hybridization. The mutant phenotype results in an overall developmental arrest which occurs at a time when the entire embryo is undergoing its most rapid phase of cellular proliferation^{24,25}. At E6.5 transcripts cannot be detected (Fig. 3c), but at E7.5 the gene is upregulated and expressed throughout the entire embryo, including the extraembryonic tissues (Fig. 3d). No difference in expression levels was detected in the three germ layers. At day E8.5, *Brca2* is expressed in the neuroepithelium and somites, with maximal expression in the migrating neural crest cells that

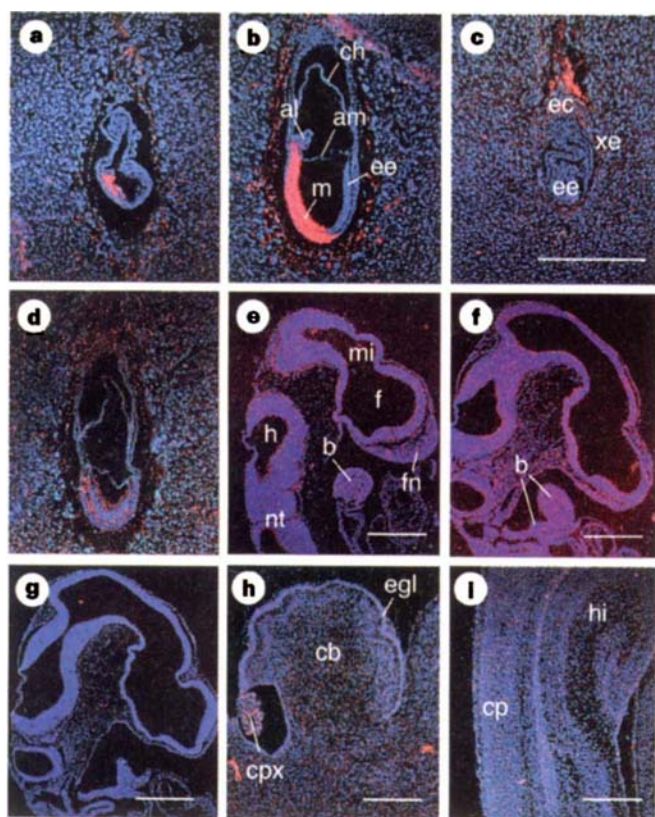


Figure 3 Expression (red signal) of *Brachyury* (*T*) (a, b) and *Brca2* (c–i) in the embryos (visualized by Hoechst 33258 DNA stain). a, *T* expression is limited to the caudalmost end of a E7.5 *brca2^{Brdm1}/brca2^{Brdm1}* embryo. b, E7.5 wild-type embryo showing expression of *T* extending throughout the posterior mesoderm. c, E6.5 wild-type embryo shows no expression of *Brca2*. d, E7.5 wild-type embryos with ubiquitous expression. e, E9.5 embryo showing broad expression in the entire neuroepithelium and in the frontonasal mesenchyme. f, E10.5 embryo illustrating a general upregulation of *Brca2* expression compared to earlier stages. g, E10.5 embryo hybridized with sense probe. At birth, cerebellum (h) and hypothalamic region (i) show no *Brca2* expression. b, Branchial arches; cb, cerebellum; cp, cortical plate; cpx, choroid plexus; egl, external granular layer; f, forebrain; fn, frontonasal mesenchyme; h, hindbrain; hi, hippocampus; m, mesoderm; mi, midbrain; nt, neural tube; other abbreviations are as in Fig. 2. Scale bars, 500 μ m.

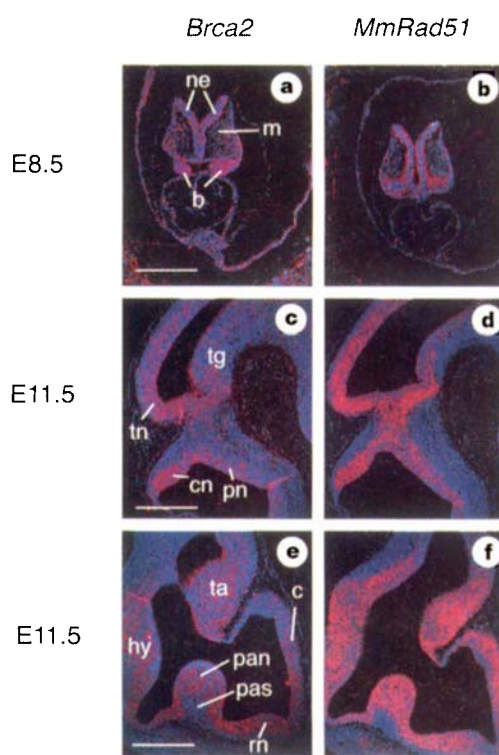


Figure 4 Coexpression of *Brca2* and *MmRad51*. a, c and e, Expression of *Brca2*; b, d and f, expression of *MmRad51*. a, b, Highly expressed *Brca2* and *MmRad51* in the neural crest cells of an E8.5 embryo which populate the branchial arches and in the neuroepithelium. c, d, Midbrain–hindbrain boundary of an E11.5 embryo. Strong expression of both genes is seen in the tectal neuroepithelium, the pontine neuroepithelium and the cerebellar neuroepithelium. Transcripts are less abundant in the tegmental neuroepithelium. e, f, Forebrain of an E11.5 embryo. Note the lower signal in the non-proliferative pallidal subventricular zone in comparison to the proliferative pallidal neuroepithelium. Expression is also detected in the hypothalamic, thalamic, cortical and rhinencephalic neuroepithelia. Note that the pial surface of the cortical neuroepithelium shows a weaker signal. b, Branchial arches; c, cortical neuroepithelium; cn, cerebellar neuroepithelium; hy, hypothalamic neuroepithelium; m, mesoderm; ne, neuroepithelium; pan, pallidal neuroepithelium; pas, pallidal subventricular zone; pn, pontine neuroepithelium; rn, rhinencephalic neuroepithelium; ta, thalamic neuroepithelium; tn, tectal neuroepithelium; tg, tegmental neuroepithelium. Scale bars, 500 μ m.

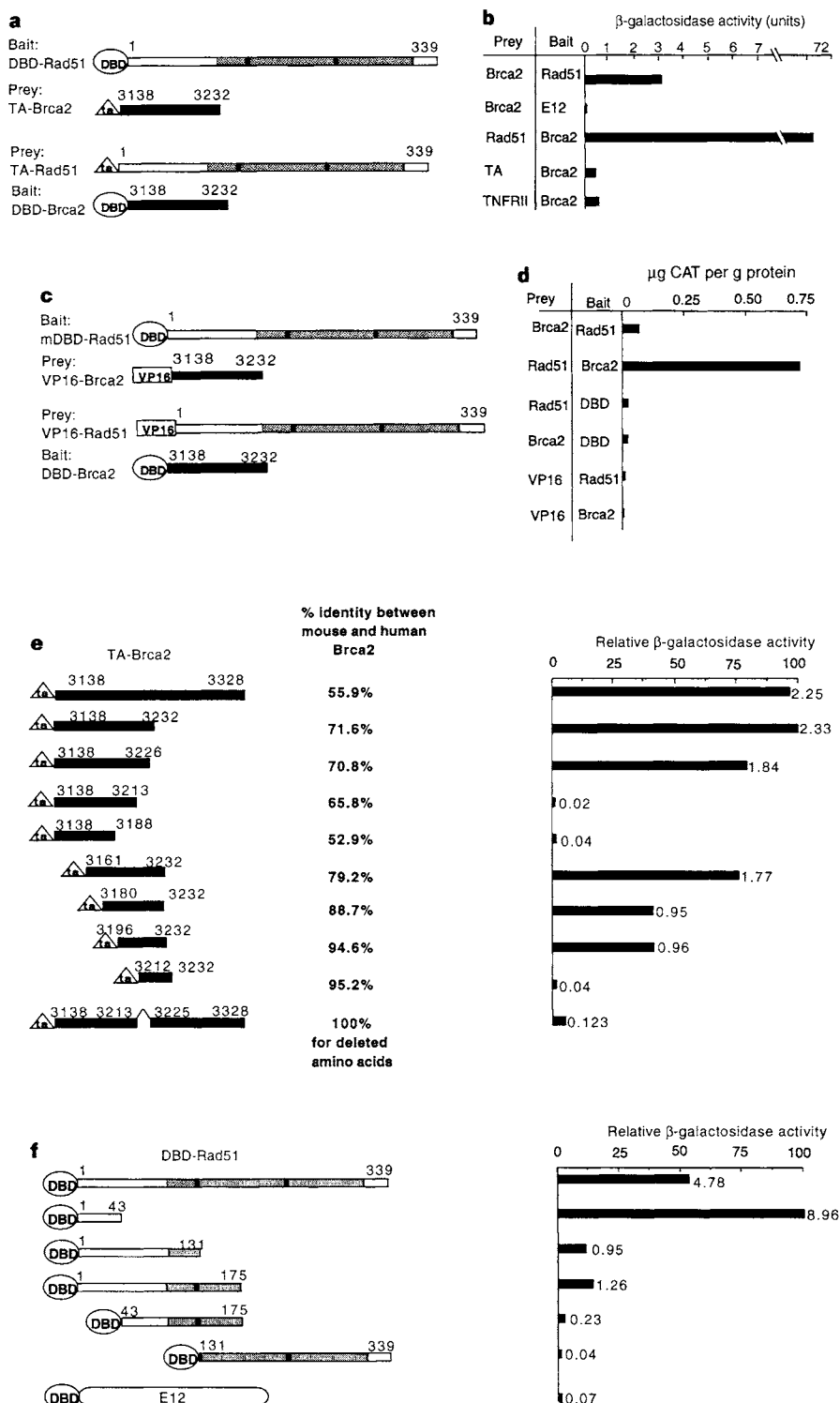


Figure 5 Two-hybrid analysis. **a**, MmRad51-Brca2 interaction in yeast cells. The bait is fused to the GAL4 DNA-binding domain (DBD) and the prey is fused to the GAL4 transactivating (TA) domain. MmRad51 (open rectangle) served as both bait, DBD-Rad51 1-339, and prey, TA-Rad51 1-339. Shaded box represents the RecA core homology region¹⁵. Dark lines represent the conserved ATP-binding motifs. The C-terminal region of Brca2 (filled rectangle) served as both bait, DBD-Brca2 3138-3232 and prey, TA-Brca2 3138-3232. **b**, β -Galactosidase activity in Miller units⁴⁶. E12 is the negative control bait. The transactivating domain of GAL4 (TA) and tumour-necrosis-factor receptor II (TNFRII) are negative control preys. **c**, MmRad51-Brca2 interaction in mammalian 3T3 cells. The baits were fused to the GAL4 DNA-binding domain (DBD) and the preys were fused to the activation domain of virion protein 16 (VP16) of herpes simplex virus. MmRad51 served as

both bait, mDBD-Rad51 1-339, and prey, VP16-Rad51 1-339. The C-terminal region of Brca2 served as both bait, mDBD-Brca2 3138-3232 and prey, VP16-Brca2 3138-3232. Association of bait and prey were measured by expression of a CAT (chloramphenicol acetyltransferase) gene with a minimal promoter from adenovirus E1b, with five consensus GLA4 binding sites (pG5 CAT from Clontech). **d**, CAT activity. Negative controls: unfused GALL4 DNA-binding domain for the bait and unfused VP16 for the prey. **e**, Deletions of Brca2. Right, per cent amino-acid identity between mouse and human Brca2. Far right, relative β -galactosidase activity after co-transfection with DBD-Rad51 1-339 as the bait. Miller units are listed at the top of each bar. **f**, Deletion analysis of MmRad51. E12 is the negative control bait. Right, relative β -galactosidase activity after co-transfection with TA-Brca2 3138-3328. Miller units are listed at the top of each bar.

populate the branchial arches (Fig. 4a). At E9.5 and E10.5, *Brca2* continues to show a similar spatial expression pattern but there is an overall increase in the level of transcript at E10.5 (Fig. 3e, f). Marked regional differences in expression are first seen by E11.5, at which time transcripts are abundant in the rhinencephalic, cortical, thalamic, hypothalamic and pallidal neuroepithelium (Fig. 4e), whereas expression in the pallidal subventricular zone and the head mesenchyme (not shown) is decreased. In the midbrain, high concentrations of transcript are detected in the inferior tectal neuroepithelium and these are lower in the superior tectal and tegmental neuroepithelium. In the hindbrain, a strong hybridization signal is seen in the pontine and cerebellar neuroepithelia (Fig. 4c). The gene is also expressed in the dorsal root ganglia and the hepatic primordium (data not shown). At the day of birth, expression in the central nervous system is downregulated to background levels (Fig. 3h, i).

We conclude that *Brca2* expression is transient and largely embryo-specific. It appears that the developmental block occurs when its expression is upregulated at around E7.5. Also, transcripts are particularly prevalent in tissues with a high mitotic index, such as the proliferating ventricular zones of the fore-, mid- and hindbrain, but transcripts are downregulated in post-mitotic cells.

Interaction between *Brca2* and *Rad51*

BRCA1 protein interacts with HsRad51, a protein likely to be involved in the repair of double-strand breaks¹³. In yeast, protein-protein interaction is essential for *ScRad51* function²⁶. To identify other proteins that might interact with MmRad51, a yeast two-hybrid screen was done with a T-cell complementary DNA library and a C-terminal region of mouse *Brca2* coding for amino acids 3,138–3,232 was isolated¹¹ (Genbank accession no. U65594) (Fig. 5a, b). This interaction was directly tested in mammalian cells with similar results (Fig. 5c, d). The association appeared to be

much stronger with MmRad51 as the prey and *Brca2* as the bait compared with MmRad51 as bait and *Brca2* as prey. The possibility that this enhanced interaction was due to transcriptional activity of *Brca2* fused to the DNA-binding domain was ruled out because in the absence of MmRad51 as prey, it showed no transactivation (Fig. 5d). This region of *Brca2* shows 72% identity with the human homologue^{5,6,11}. As the overall identity between the two proteins is 59%, the relatively high degree of conservation in this region suggests that it is functionally significant¹¹. A deletion analysis was done on this *Brca2* region to determine the amino acids that are critical for the association with MmRad51 (Fig. 5e). Deletions of *Brca2* prey were individually cotransformed into yeast cells with MmRad51 bait and β -galactosidase was assayed. None of the *Brca2* deletions interacted with a negative control bait, E12 (not shown). The minimal region that showed a strong association was a 36-amino-acid fragment (residues 3,196–3,232). Deleting 13 of these amino acids (residues 3,213–3,226) ablated the interaction. Note, however, that these 13 amino acids may not be directly involved in *Brca2*–MmRad51 interaction as the absence of these amino acids may alter the folding of the mutant protein and thus prevent the two proteins from binding. The 36 and 13 amino acids are 95% and 100% conserved, respectively, between mouse and human *Brca2*, suggesting that this region has a critical function.

A deletion analysis was also done with the MmRad51 bait to determine the region critical for the association with *Brca2*. The N-terminal region of MmRad51 (amino acids 1–43), but not the C-terminal region, was essential for the interaction (Fig. 5f). The N-terminal region is also responsible for *ScRad51*–protein associations in *S. cerevisiae*²⁶, suggesting that this region has a conserved function from yeast to mammals.

Hypersensitivity to γ -irradiation

Mmrad51 and *Brca2* homozygous mutants arrest development at a similar developmental stage and the gene products interact¹⁷ (see above). If *Brca2* and MmRad51 interact with each other in the embryo then they should be comparably expressed. The expression pattern of the two genes was found to be almost identical (Fig. 4). Both genes are expressed in rapidly dividing cells and are down-regulated in non-dividing cells.

MmRad51 homozygous mutant embryos are hypersensitive to ionizing radiation¹⁷. This phenotype should be seen in *Brca2* mutants if the *Brca2*/Rad51 association has functional significance. Given that it was not possible to make *brca2*^{Brdm1}/*brca2*^{Brdm1} cell lines, *brca2*^{Brdm1}/*brca2*^{Brdm1} and control (+/+, *brca2*^{Brdm1}/+) E3.5 embryos were investigated for hypersensitivity to γ -radiation. Embryos were obtained from *brca2*^{Brdm1}/+ intercrosses and grown *in vitro* for seven days. Without exposure to radiation, the outgrowth of the inner cellular mass and the number of trophoblast giant cells was equivalent for both mutant and control embryos (Fig. 6a, b). Therefore, inner cell mass outgrowth and trophoblast cell number can be compared for radiation sensitivity. Individual blastocysts were genotyped by PCR (Fig. 6e).

Survival and outgrowth of the inner cell mass was observed for embryos exposed to 400 Rads of γ -radiation. Inner cell mass outgrowth was marginally reduced for eight control (wild-type and heterozygous) embryos after exposure to 400 Rads (Fig. 6c). However, the inner cell mass of *brca2*^{Brdm1}/*brca2*^{Brdm1} embryos was totally ablated after 400 Rads in eight out of eight embryos (Fig. 6d). In addition to the inner cell mass outgrowth, γ -radiation reduced the number of trophoblast cells in the homozygous mutants, whereas in cells derived from wild-type and *brca2*^{Brdm1}/+ embryos, there was only a slight decrease in the number of trophoblast cells. In unirradiated control embryos, there was an average of 35 ± 7 trophoblast cells ($n = 13$, whereas irradiated control embryos had 31 ± 5 trophoblast cells ($n = 8$)). However, in the case of *brca2*^{Brdm1}/*brca2*^{Brdm1} embryos, the number of trophoblast cells was reduced to 15 ± 3 ($n = 8$).

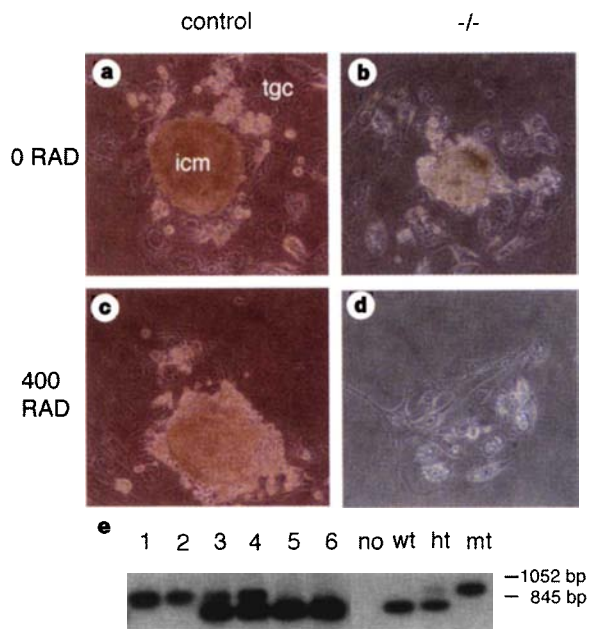


Figure 6 Results of *in vitro* culture of blastocysts after exposure to 400 Rads of γ -radiation. Blastocysts were observed after 7 days in culture. **a**, Unirradiated control blastocyst; **b**, unirradiated mutant blastocyst; **c**, radiated control; and **d**, radiated mutant blastocyst. **e**, Genotyping of blastocysts. Lanes: 1, 2, homozygous mutant; 3, 4, heterozygous; 5, 6, wild type; next four lanes are controls: no, no DNA; wt, wild-type AB2.2 DNA; ht, heterozygous ES cell (D6) DNA; mt, pBrca2TV DNA. icm, Inner cell mass; tgc, trophoblast giant cells. Scale bars, 120 μ m.

Discussion

Brca2-deficient embryos suffer an overall developmental arrest after 6.5 days of gestation. This occurs around the time when *Brca2* becomes detectable by *in situ* hybridization analysis. The inability to isolate ES cells homozygous for *Brca2* mutation and the association of embryonic expression of *Brca2* with cells with a high mitotic index is consistent with a *Brca2* function in cell proliferation. This conclusion is supported by studies showing that *Brca2* expression is tightly regulated during mammary epithelial proliferation and differentiation, which is coordinated with *Brca1* expression¹². Furthermore, *Brca2* is expressed in a cell-cycle-dependent manner and peaks at the G₁/S boundary.

The homozygous mutant phenotype of *Brca2* is similar to that of *Brca1* and *Mmrads1* mutant phenotypes, so these genes may function in similar pathways^{17,27,28}. *Brca2*, *Brca1* and *Mmrads1* mutants arrest during development at similar times and homozygous mutant ES cells could not be isolated for any of these genes^{17,27,28}. In some respects, the *Brca2* mutant embryos do not show a defect in cell proliferation in *in vitro* cultures of implanting blastocysts (Fig. 6a, b), whereas *Brca1* and *Mmrads1* mutants fail to proliferate^{17,27,28}. In addition, *Brca2* mutant embryos initiate mesoderm formation which is absent in *Brca1* and *Mmrads1* mutant embryos. However, radiation sensitivity assays show that *Brca2* mutants, like *Mmrads1* mutants, are hypersensitive to γ -irradiation, implying that the cell-proliferation defect may be secondary to a response to a defect in DNA repair. *Mmrads1* mutant embryos appear to be more sensitive to γ -irradiation than *Brca2* mutants based on the sensitivity of trophoblast cells.

Colocalization and coimmunoprecipitation have shown that human BRCA1 protein interacts with HsRad51 (ref. 13). We have used two-hybrid data from both yeast and mammalian cells to define a mouse *Brca2*/Rad51 interaction. The stoichiometry for this interaction remains to be determined and the existence of additional components or of a conserved mediator in a *Brca2*/MmRad51 complex cannot be ruled out. The functional significance of this interaction is supported by the similarity of the *Mmrads1* and *Brca2* mutant phenotypes, by the precise coexpression of the two genes, and by the sensitivity of *Brca2* deficient-embryos to γ -irradiation.

This association of Rad51 with *Brca1* and *Brca2* and the resultant sensitivity of *Mmrads1*- and *Brca2*-deficient cells to irradiation may explain the high penetrance of early-onset cancer phenotypes exhibited by patients with either *BRCA1* or *BRCA2* mutations^{1,3}. In mammary epithelial cells that have lost *BRCA1/2* activity, the HsRad51-mediated DNA-repair functions are presumably compromised, which destabilizes the genome. Rad51 thus serves to suppress tumour formation through interaction with both *Brca1* and *Brca2*. This raises the possibility that *Rad51* might also be a tumour-suppressor gene. In humans, *HsRad51* maps to chromosome 15q15.1, which shows loss of homozygosity in breast tumours^{29,30} but no mutation analysis of *HsRad51* in these tumours has been reported.

The role of *Brca2* in DNA repair is unknown; it is a very large protein and so could have several functions, including that of a scaffold protein or a matchmaker. This role would be consistent with the greater severity of the *Mmrads1* mutation compared with that in *Brca2*; presumably in the absence of *Brca2*, some *Mmrads1* functions can continue, but eventually the auxiliary function of *Brca2* becomes limiting. Many proteins and interactions are essential for monitoring and for repairing DNA breaks in conjunction with regulating the cell cycle in response to these breaks³¹. A defect in the repair pathway would lead to cell-cycle arrest or cell death.

Why does *Brca2* deficiency cause cell lethality in early embryos and ES cells, whereas *BRCA2*-deficient mammary epithelial cells proliferate and form tumours? One explanation is that the proliferation arrest is cell-type specific. The embryo and totipotent ES cells are very sensitive to unrepaired breaks and so the cells are not viable. In contrast, *BRCA2*-deficient breast epithelial cells can

survive with an unstable genome, but presumably they accumulated genomic alterations that contribute to the malignancy. Taken together, *Brca1*, *Rad51* and *Brca2* may be involved in detecting and repairing double-strand breaks, thereby controlling cell-cycle progression. Loss of this repair/monitor system has a direct effect on cell proliferation in the early embryo and tumorigenesis in the adult.

Mouse knockouts of familial tumour-suppressor genes vary in the degree to which they model the human syndrome. In some models, tumours are not observed^{27,28,32}, in others the tissue spectrum is quite different³³, and others closely reflect the human disease^{34–37}. Evidently there are many species-specific factors that contribute to the probability of tumour growth in *Brca2* heterozygous mice. It is too early to define the extent to which *Brca2* heterozygous mice will resemble the susceptibility, penetrance and tissue specificity of the disease in humans. Given the conservation of the *Rad51* and the portion of *Brca2* involved in the interaction between the two species and the sensitivity of *Brca2*-deficient cells to γ -irradiation, it is likely that the heterozygous mice will be susceptible to tumours. □

Methods

Construction of *Brca2* targeting vectors. A targeting vector with positive-negative selection designed to delete 2.8 kb of the *Brca2* genomic locus, including the splice acceptor site and 812 amino acids of exon 11. A genomic clone was isolated from a mouse 129SvEv genomic library using a mouse *Brca2* cDNA (Fig. 1a). The targeting vector (pBrca2TV) consisted of a 3.2 kb *EcoRI*–*SalI* (*SalI* site from polylinker of phage clone) fragment as the 5' homology region and a 3.0-kb *EcoRI* fragment representing the 3' homology region which were ligated to the human PGK–*Hprt* minigene (Fig. 1b). The HSV *tk* gene was ligated downstream of the 3' homology region. pBrca2-puroTV was constructed similarly to pBrca2TV, except that the puromycin-resistance gene, *puro*, was used as positive-selection marker (Fig. 1e). Electroporation and ES cell culture were done as described³⁸.

Southern blot analysis to genotype ES cells and mice. To identify the mutant ES cells that were correctly targeted and to genotype the mice, genomic DNA was digested with *Bam*HI and analysed as described³⁹. An 800-bp *Eco*RI fragment was used that detects a 9.5-kb fragment in wild-type DNA and 14.0-kb fragment in targeted clones (Fig. 1d). Homologous recombination in the 5' homology region was confirmed by stripping the blot and rehybridizing with a 400-bp *Rsa*I fragment. The wild-type *Bam*HI fragment detected by this probe is 3.5 kb and the mutant fragment is 14 kb (data not shown). To identify the second targeting event in D6 cells electroporated with pBrca2puroTV, Southern blots of *Bam*HI-digested genomic DNA were hybridized with the 800-bp *Eco*RI fragment. The probe detects a 9.5-kb wild-type fragment, a 14-kb mutant band from the first targeting and a new 8.5-kb mutant fragment from retargeting (Fig. 1g). Mice were generated from ES cells as described³⁸.

Histological analysis and *in situ* hybridization. Embryos contained inside the decidua were fixed in Bouin's solution and processed for histological analysis as described²³. Sections were stained with haematoxylin and eosin. *In situ* hybridization was done as described⁴⁰. *Brca2* antisense and sense RNA probes corresponding to the nucleotides 6,887 and 7,249 were used. Hybridization was overnight at 58 °C and washes were at 64 °C. *Brachyury* and *Mmrads1* expression were detected as described^{15,41}.

Yeast and mammalian two-hybrid vectors. Construction of the *Mmrads1* bait, DBD–Rad51 1–339: Full-length *Mmrads1* cDNA was amplified by the polymerase chain reaction (PCR) with *pfu* DNA polymerase (Stratagene) using a 5' sense oligonucleotide with a *Bam*HI site (5'-AAGGATCGATGGATCCTCATGGCTATGCAAAATG-3') and a 3' antisense oligonucleotide with a *Kpn*I site (5'-CAGATTCATGGTACCTTTGGCATCGCC-3'). The amplified PCR fragment was cloned into the bait expression vector pAS1 (ref. 42).

Deletions of the *Mmrads1* bait. The PCR fragment was digested with *Bam*HI/*Hae*II, *Bam*HI/*Eco*RI, and *Bam*HI/*Nhe*I and cloned into pAS1 to create DBD–Rad51 1–93, DBD–Rad51 1–131, and DBD–Rad51 1–175, respectively. The other three deletion mutants were made from PCR products generated with *pfu* DNA polymerase using the following primers: DBD–Rad51 1–43, 5' sense, 5'-CATATGGCCATGGAGATGGCTATGCAAAATG-3', 3' antisense 5'-GGATCCGTCGACCCACTGTATGGTACCCGG-3'; DBD–Rad51 43–175, 5'

sense 5'-CATATGGCCATGGAGGCTGTTGCTTATGCACGG-3', 3' antisense 5'-GGATCCGTCGACTGCTAGCAGCCGCTCCGG-3'; DBD-Rad51 131-339, 5' sense 5'-CATATGGCCATGGAGTTTGGAGAATTCCGAAC-3', 3' antisense 5'-GGATCCGTCGACGAGTCAGTCTTTGGCATC-3'.

Deletions of the *BRCA2* prey. A *Brca2* cDNA fragment encoding amino acids 3,138-3,232 was isolated from the yeast two-hybrid screen. TA-*Brca2* 3138-3328 was obtained by a three-way ligation with pACT cut with *XhoI*-*HindIII*, *Brca2* cut with *XhoI*-*StyI* (from TA-*Brca2* 3138-3322) and *Brca2* cut with *StyI*-*HindIII* (from a genomic DNA library; extends the original *Brca2* cDNA to the stop codon). From these template DNAs, deletion clones were made by PCR and cloned into the *XhoI* site of pACT. Oligonucleotides used were: TA-*Brca2* 3138-3226, 5' sense 5'-CTATCTATTCGATGATGAAGA-3' (PACTUP for pACT), 3' antisense, 5'-CACCTCGAGCTCCGTCGGGCTGAAAAG-3'; TA-*Brca2* 3138-3213, 5' sense, PACTUP, 3' antisense, 5'-CACCTCGAGCTCCGTCGGGCTGAAAAG-3'; TA-*Brca2* 3138-3188, 5' sense, PACTUP, 3' antisense, 5'-ACAGGTTTGGGTCATCAA-3'; TA-*Brca2* 3161-3232, 5' sense, 5'-GAAGTCTATGCCCTGGCTCA-3', 3' antisense, 5'-GTGAACCTTGGGGGTTTTCAGT-3' (PACTLW for pACT); TA-*Brca2* 3180-3232, 5' sense, 5'-GAACGAGATTGATGACCCAA-3', 3' antisense, PACTLW; TA-*Brca2* 3196-3232, 5' sense, 5'-CACCTCGAGATTACCTTTCCTAAGTCGGCTGCCCTTA-3', 3' antisense, PACTLW; TA-*Brca2* 3212-3232, 5' sense, 5'-CACCTCGAGATTACCTTGTCTCTCCAGCTGCACA-3', 3' antisense, PACTLW; TA-*Brca2* del 3213-3225, template; TA-*Brca2* 3138-3328, first PCR 5' part was same as TA-*Brca2* 3138-3213; for 3' part, 5' sense, 5'-TCCCATCTGTACCTTTGTCTAGATCTTGTGGCAGAAATACGCA-3', 3' antisense, PACTLW. These fragments were linked by second PCR with 5' PACTUP and 3' PACTLW primers.

To make DBD-*Brca2* 3138-3232, the *BRCA2* C-terminal region encoding amino acids 3138-3232 was released from TA-*Brca2* 3138-3232 with *BglII* and cloned into the *BamHI* site of pAS1.

Yeast two-hybrid screen. HF7c yeast⁴³ cells containing DBD-Rad51 were transformed with 400 µg mouse T cell pACT library. MmRad51 interacting clones were identified as described^{42,44}. Colonies were tested for specificity by transforming HF7c yeast without bait and with a nonspecific bait, E12. For deletion analysis, the bait and prey were cotransformed and grown in the absence of tryptophan and leucine. β-Galactosidase activity in yeast was assayed as described⁴⁵.

Mammalian two-hybrid screen. 3T3 cells were transiently cotransfected with bait, prey and reporter plasmids by lipofection (Lipofectamine, Gibco BRL). Sixty hours later, CAT enzyme was extracted from the cells and measured by ELISA (Boehringer).

In vitro culture and radiation treatment of blastocysts. Blastocysts were isolated at day 3.5 from *brca2*^{Brdm1}/+ females mated to *brca2*^{Brdm1}/+ males (day zero). Blastocysts were exposed to 400 Rads of γ-radiation in a Gammacell 1000 irradiator (1,000 Rads min⁻¹) and cultured in DMEM + 15% FCS-containing medium for 7 days.

PCR genotyping of blastocysts. Blastocysts were picked after washing the plates with PBS and lysing in 20 µl lysis buffer (50 µl KCl, 10 mM Tris HCl, pH 8.3, 2.5 mM MgCl₂, 0.01 mg ml⁻¹ gelatine, 0.45% Tween 20 and 0.45% NP40) and 2 µl 10 mg ml⁻¹ Proteinase K for 4-5 h at 55 °C. Three primers were used in the PCR reaction. A single antisense primer (5'-CCCACTAGCTGTATGAAAAC-3') was designed to amplify both wild-type as well as the mutant band. The first sense primer (5'-GCAAAAAGTAGGACCAAGAGG-3') was designed from exon 11 to amplify an 845-bp wild-type band and a second sense primer from human *Hprt* minigene (5'-CCCACGAAGTGTGGATATA-3') amplified a 1,052-bp mutant band. PCR products were resolved on 1.2% agarose gel. Southern blots were hybridized with [γ-³²P]ATP-labelled oligomer (5'-TACTGATTGCTTTCTTGTA-3').

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1. Wooster, R. *et al.* Localization of a breast cancer susceptibility gene, *BRCA2*, to chromosome 13q12-13. *Nature* **265**, 2088-2090 (1994).
2. Smith, S. A., Easton, D. G., Evans, D. G. R. & Ponder, B. A. J. Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild type chromosome. *Nature Genet.* **2**, 128-131 (1992).
3. Easton, D. F., Bishop, D. T., Ford, D., Crookford, G. P. & the breast cancer linkage consortium Genetic linkage analysis in the familial breast and ovarian cancer: Results from 214 families. *Am. J. Hum. Genet.* **52**, 678-701 (1993).
4. Gayther, S. A. *et al.* Variations of risks of breast and ovarian cancer associated with different germline mutations of the *BRCA2* gene. *Nature Genet.* **15**, 103-105 (1997).

5. Wooster, R. *et al.* Identification of the breast cancer susceptibility gene *BRCA2*. *Nature* **378**, 789-792 (1995).
6. Tavtigian, S. V. *et al.* The complete *BRCA2* gene and mutations in chromosome 13q-linked kindreds. *Nature Genet.* **12**, 333-337 (1996).
7. Couch, F. J. *et al.* *BRCA2* germline mutations in male breast cancer cases and breast cancer families. *Nature Genet.* **13**, 123-125 (1996).
8. Neuhausen, S. *et al.* Recurrent *BRCA2*617delT mutations in Ashkenazi Jewish women affected by breast cancer. *Nature Genet.* **13**, 126-128 (1996).
9. Phelan, C. M. *et al.* Mutation analysis of the *BRCA2* gene in 49 site specific breast cancer families. *Nature Genet.* **13**, 120-122 (1996).
10. Thorlacius, S. *et al.* A single *BRCA2* mutation in male and female breast cancer families from Iceland with varied cancer phenotypes. *Nature Genet.* **13**, 117-119 (1996).
11. Sharan, S. K. & Bradley, A. Murine *Brca2*: Sequence, map position and expression pattern. *Genomics* **40**, 234-241 (1997).
12. Rajan, J. V., Wang, M., Marquis, S. T. & Chodosh, L. A. *Brca2* is coordinately regulated with *Brca1* during proliferation and differentiation in mammary epithelial cells. *Proc. Natl Acad. Sci. USA* **93**, 13078-13083 (1996).
13. Scully, R. *et al.* Association of *BRCA1* with *Rad51* in mitotic and meiotic cells. *Cell* **88**, 265-275 (1997).
14. Malkova, A., Ivanov, E. L. & Harber, J. E. Double strand break repair in the absence of *RAD51* in yeast: a possible role of break-induced DNA replication. *Proc. Natl Acad. Sci. USA* **93**, 7131-7136 (1996).
15. Shinohara, A. *et al.* Cloning of human, mouse and fission yeast recombination genes homologous to *RAD51* and *RecA*. *Nature Genet.* **4**, 239-243 (1993).
16. Shinohara, A., Ogawa, H. & Ogawa, T. *Rad51* protein involved in repair and recombination in *S. cerevisiae* is a *RecA*-like protein. *Cell* **69**, 457-470 (1992).
17. Lim, D.-S. & Hastay, P. A. Mutation in mouse *rad51* results in an early embryonic lethal that is suppressed by a mutation in *p53*. *Mol. Cell. Biol.* **16**, 7133-7143 (1996).
18. Sung, P. & Roberson, D. L. DNA strand exchange mediated by a *Rad51*-ssDNA nucleoprotein filament with polarity opposite to that of *RecA*. *Cell* **82**, 453-461 (1995).
19. Radding, C. M. Helical interactions in homologous pairing and strand exchange driven by *RecA* protein. *J. Biol. Chem.* **266**, 5355-5358 (1991).
20. Baumann, P., Benson, F. E. & West, S. C. Human *Rad51* protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*. *Cell* **87**, 757-766 (1996).
21. Bishop, D. K. *RecA* homologs *Dmc1* and *rad51* interact to form multiple nuclear complexes prior to meiotic chromosome synapsis. *Cell* **79**, 1081-1092 (1994).
22. Rockmill, B., Sym, M., Schertham, H. & Roeder, G. S. Role of two *RecA* homologs in promoting meiotic chromosome synapsis. *Genes Dev.* **9**, 2684-2695 (1995).
23. Kaufman, M. H. In *The Atlas of Mouse Development* 2-5 (Academic, San Diego, 1992).
24. Snow, M. L. H. Gastrulation in the mouse: Growth and regionalization of the epiblast. *J. Embryol. Exp. Morph.* **42**, 293-303 (1977).
25. Power, M.-A. & Tam, P. P. L. Onset of gastrulation, morphogenesis and somitogenesis in mouse embryos displaying compensatory growth. *Anat. Embryol.* **187**, 493-504 (1993).
26. Donovan, J. W., Milne, C. T. & Weaver, T. D. Homotypic and heterotypic protein associations control *Rad51* function in double-strand break repair. *Genes Dev.* **8**, 2552-2562 (1994).
27. Hakem, R. *et al.* The tumor suppressor gene *Brcal* is required for embryonic cellular proliferation in the mouse. *Cell* **85**, 1009-1023 (1996).
28. Liu, C. Y., Flesken-Nikitin, A., Li, S., Zeng, Y. & Lee W.-H. Inactivation of the mouse *Brcal* gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development. *Genes Dev.* **10**, 1835-1843 (1996).
29. Devilee, P. *et al.* Allelotype of human breast carcinoma: a second major site for loss of heterozygosity is on chromosome 6q. *Oncogene* **6**, 1705-1711 (1991).
30. Wick, W. *et al.* Evidence for a novel tumor suppressor gene on chromosome 15 associated with progression to a metastatic stage in breast cancer. *Oncogene* **12**, 973-978 (1996).
31. Carr, A. M. & Hoekstra, M. F. The cellular response to DNA damage. *Trends Cell Biol.* **5**, 32-40 (1995).
32. Kreidberg, J. A. *et al.* WT-1 is required for early kidney development. *Cell* **74**, 679-791 (1993).
33. Hu, N. *et al.* Heterozygous *Rb-1 delta20/+* mice are predisposed to tumors of the pituitary gland with nearly complete penetrance. *Oncogene* **9**, 1021-1027 (1994).
34. Su, L.-K., Vogelstein, B. & Kinzler, K. W. Association of the APC tumor suppressor protein with catenins. *Science* **262**, 1734-1737 (1993).
35. Sands, A. T., Abuin, A., Sanchez, A., Conti, C. J. & Bradley, A. High susceptibility to ultraviolet-induced carcinogenesis in mice lacking *XPC*. *Nature* **377**, 162-165 (1995).
36. de Vries, A. *et al.* Increased susceptibility to ultraviolet B and carcinogenesis of mice lacking the DNA excision repair gene *XPA*. *Nature* **377**, 169-173 (1995).
37. Donehower, L. A. *et al.* Mice deficient for *p53* are normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221 (1992).
38. Matzuk, M. M., Finegold, M. J., Su, J.-G. J., Hsueh, A. J. W. & Bradley, A. α-Inhibin is a tumour-suppressor gene with a gonadal specificity in mice. *Nature* **360**, 313-319 (1992).
39. Ramirez-Solis, R., Davis, A. & Bradley, A. Gene targeting in embryonic stem cells. *Methods. Enzymol.* **225**, 855-878 (1993).
40. Albrecht, U., Eichele, G., Helms, J. A. & Lu, H.-C. in *Molecular and Cellular Methods in Developmental Toxicology* (ed. Daston, G. P.) 23-48 (CRC Press, Boca Raton, 1997).
41. Herrmann, B. G. Expression pattern of the *Brachyury* gene in whole-mount *T^{wt}/T^{wt}* mutant embryos. *Development* **120**, 913-917 (1991).
42. Durfee, T. *et al.* The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7**, 555-569 (1993).
43. Feilottter, H. E., Hannon, G. J., Ruddell, C. J. & Beach, D. Construction of an improved host strain for two hybrid screening. *Nucleic Acids. Res.* **22**, 1502-1503 (1994).
44. Breeden, L. & K. Nasmyth, K. Regulation of the yeast *HO* gene. *Cold Spring Harb. Symp. Quant. Biol.* **50**, 643-650 (1985).
45. Guarente, L. Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Meth. Enzymol.* **101**, 181-191 (1983).
46. Miller, J. H. in *Experiments in Molecular Genetics* 352-355 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972).

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Bi-directional plasma jets produced by magnetic reconnection on the Sun

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Magnetic reconnection, the process by which magnetic lines of force break and rejoin into a lower-energy configuration, is considered to be the fundamental process by which magnetic energy is converted into plasma kinetic energy¹. The Sun has a large reservoir of magnetic energy, and the energy released by magnetic reconnection has been invoked to explain both large-scale events, such as solar flares^{2,3} and coronal mass ejections⁴, and small-scale phenomena, such as the coronal and chromospheric microflares that probably heat and accelerate the solar wind^{5,6}. But the observational evidence for reconnection is largely indirect, resting on observations of variations in solar X-ray morphology and sudden changes in the magnetic topology^{7,8}, and on the apparent association between some small-scale dynamic events and magnetic bipoles^{9,10}. Here we report ultraviolet observations of explosive events in the solar chromosphere that reveal the presence of bi-directional plasma jets ejected from small sites above the solar surface. The structure of these jets evolves in the manner predicted by theoretical models of magnetic reconnection^{11,12}, thereby lending strong support to the view that reconnection is the fundamental process for accelerating plasma on the Sun.

The solar network is the pattern of supergranulation cells seen over the entire surface of the Sun. The cell boundaries, along which the magnetic flux concentrates, is in a state of continuous variability and violent activity. Sudden small-scale plasma jets, microflares^{9,13} and explosive events^{14–16} are examples of this activity. Explosive events, the subject of this Letter, were first seen in the ultraviolet spectra of the Naval Research Laboratory's high-resolution telescope and spectrograph (HRTS) flown on several rocket flights and on Spacelab 2^{14,17}. They were found to be short-lived (60 s), small-scale (1,500 km), high-velocity ($\pm 150 \text{ km s}^{-1}$) flows that occur very frequently over the entire solar surface¹⁶. There are estimated to be over 30,000 events at any one time on the Sun. The energy involved, however, suggests that they are not the major source of mass or energy in either the chromosphere or corona^{15,16}. Their importance lies in the fact that they probably represent the high-energy tail of a spectrum of network events that occur on scales unobservable with current techniques. Dere *et al.*¹⁸ noted that explosive events are associated with freshly emerged magnetic field and that their Doppler velocities are roughly equal to the Alfvén speed in the chromosphere. They concluded that the events result from magnetic reconnection. They also speculated on the possible bi-directional nature of the flows; however the structure of the flows could not be resolved due to limited time and space coverage. The ultraviolet spectrometer SUMER^{19–21} (the Solar Ultraviolet Measurements of Emitted Radiation instrument) on the spacecraft SOHO (the Solar and Heliospheric Observatory) has recently made it possible to observe the chromospheric network continuously over an extended period and to discern the spatial structure of the flows associated with these explosive events. Here we show three examples of explosive events in which the bi-directional nature of the jets is clearly seen. In all cases (1) the Doppler shifts change from red to blue within a few arcseconds, (2) the emission is brightest at the position where the shifts change sign, and (3) the two wings of the emission move away from the site of brightest emission across the Sun's surface.

SUMER is a high-resolution telescope and spectrometer designed to obtain line profiles with a wavelength resolution of $\sim 40 \text{ mÅ}$ over the wavelength range 456–1,610 Å and with a spatial resolution of $\sim 1 \text{ arcsec}$ (715 km on the Sun) along the entrance slit which is aligned north–south. Explosive events are so frequent that even with the smallest SUMER spectrometer slit, which only covers $\sim 10^{-5}$ of the solar surface, at least one event every five minutes is almost certain to be seen. Events have been detected in several spectral lines from ions of singly to seven-times-ionized species. They therefore cover a wide range of temperatures, but appear to be most prominent in the Si iv 1,393, 1,402 Å line pair formed at $\sim 10^5 \text{ K}$.

Figure 1 shows sequences of the Si iv 1,393 Å profile obtained during two 90-min observing periods on the 21 and 23 June 1996. The rasters consisted of consecutive 5-s exposures at six or eight scan positions separated by 1.1 arcsec in the east–west direction. In each scanning sequence, first the even scan positions were measured, then the odd positions. During the time of a complete scan, the intensity at the same position changes smoothly enough to justify temporal intensity interpolation at the missing scan position. With the instrument slit orientated north–south, an area of $\sim 9'' \times 120''$ ($\sim 10^{-4}$ of the solar surface) was surveyed with a resolution of about 1 arcsec in both directions and a repetition rate of about 3 per minute.

The first example (Fig. 1a) is from a sequence of rasters taken at solar latitude $\sim 60^\circ$, near the central meridian. Hence the observed line-of-sight velocities are inclined with an angle of 60° to the Sun's surface normal. We see an explosive event lasting about 4 minutes. In the first 60 s, broad profiles with a distinct blue asymmetry are seen at just three scan positions. After 90 s the Doppler shifts have reached a maximum and the event size has almost doubled in the east–west direction. Now the profiles change from predominantly red shifts on the left to predominantly blue shifts on the right. Over the final 90 s the event seems to fade uniformly along its extent. Throughout the whole event, its centre, as identified from the sign change in the Doppler shift, moves less than 1 arcsec. This central position is also the brightest in this emission line.

The observed emission-line asymmetries are those expected from a bi-directional jet whose flow axis is directed at some angle to the line of sight, as sketched in Fig. 2a. As the slit is moved across the jet, the line profiles change from an asymmetric red-shifted profile on the left to a symmetric profile at the centre of the jet and finally to an asymmetric profile with a blue shift on the right. The centre of the jet may be identified with the source of the jet as it is also the position where we see the event first and it stays the brightest throughout the growth phase.

During the initial 30–90 s of the event its size increases by 1.1 arcsec to the left and right. If we assume that this is caused by the heads of the jet propagating out from its source then it is possible to obtain a rough estimate of the true length of the jet. The apparent motion of the heads of the jets during the initial phase corresponds to a transverse velocity of $15\text{--}30 \text{ km s}^{-1}$. If the true velocity of the jet head is of the order of the Doppler shift, 100 km s^{-1} , then the inclination angle must be $\sim 10^\circ$. From the apparent length ($4 \times 10^3 \text{ km}$), we can conclude that the true jet length is $(1.2\text{--}2.4) \times 10^4 \text{ km}$. The jet probably covers much of the chromosphere and even reaches out into the lower corona. Its final length is comparable to that obtained for macrospicules seen in the ultraviolet on the limb²². The lifetime, however, is a little short for a macrospicule although one macrospicule with a lifetime of 3 min has been reported²². The event lasts for about two or three minutes after its initial growth phase and it does not continue to spread in size beyond about 6 arcsec. Also, the centre of the jet seems to stay very localized compared to the high gas velocities that it produces.

The second example (Fig. 1b) is from a sequence of rasters taken at disk centre. The observed line-of-sight velocities are therefore normal to the solar surface. This event lasts for $\sim 4.5 \text{ min}$. After the

jet has evolved, the blue stream of the jet is far more extended than the red stream. Again we see a spatial spreading of the event during the initial two minutes. During its decay, the velocities remain large as long as the event is bright enough to be seen. In this case, the intensity at the centre of the jet disappears earlier than the intensity in the blue stream of the jet. During this 4.5-min period and within

the same $9'' \times 40''$ area we see the blue stream of a second jet (some distance along the slit) that flared up and faded during the evolution of the first jet. This illustrates how very frequently these jets occur.

The third example (Fig. 1c), also observed at disk centre, shows a jet that is orientated with the asymmetric blue profiles on the left and the red-shifted profiles on the right. The two streams of the jet

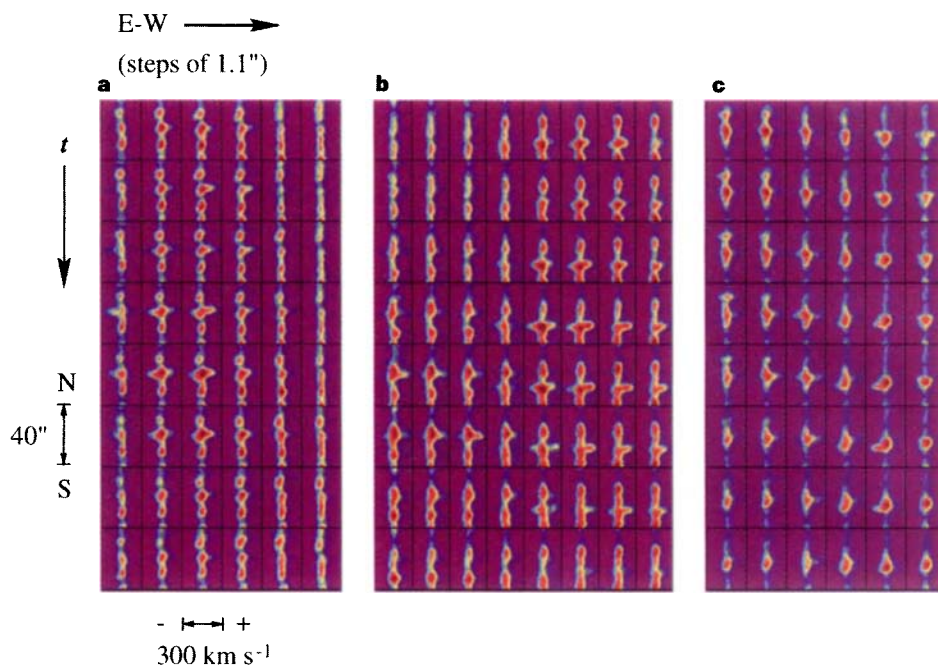


Figure 1 The jets' evolution seen in the Si IV 1,393-Å line profiles obtained with the high-resolution spectrometer SUMER on board SOHO. Each unit in the figures shows a 40-arcsec section of a spectrum along the slit for Doppler velocities from -150 km s^{-1} (red) to $+150 \text{ km s}^{-1}$ (blue). Red shifts are to the left and blue shifts are to the right. Each row shows a single spatial raster with a step size corresponding to a 1.1-arcsec displacement of the slit in the east-west direction. Time increases from top to bottom. **a**, The time between rows is 30 s. The event starts, and is centred at, column 3. The jet has propagated ~ 1 arcsec to the left and right in the 30 s between taking rasters in rows 2 and 3. The shift from red to

blue line asymmetry is clearly visible in rows 4 and 5. **b**, The time between rows is 40 s. The main event is centred in column 5. There is a second event centred in column 1 that is first noticeable in row 4 and lasts ~ 2 min. In the main event, the blue stream extends over at least three positions and red stream over only one position. We note that the emission from the jet source (column 5) fades before that from the head of the blue stream (columns 7 and 8). **c**, The interval between rows is 20 s. The event is centred in column 4 and reaches its full extent by row 3. This jet is orientated so that the blue stream is encountered before the red stream. Both streams are offset from each other by ~ 8 arcsec along the slit.

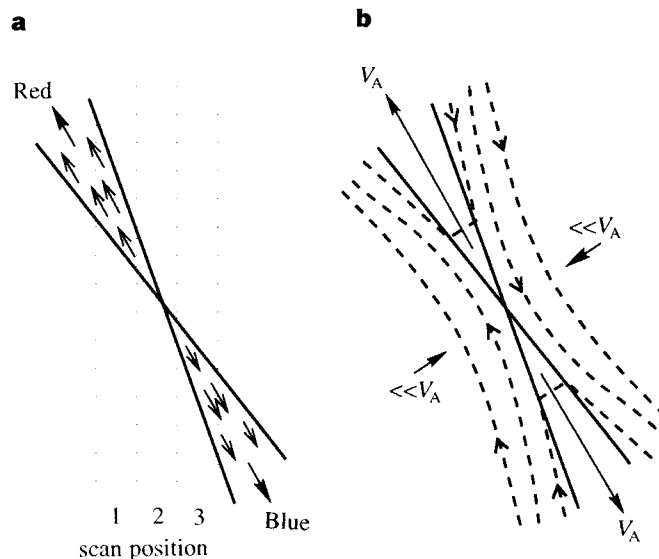


Figure 2 A schematic sketch of a bi-directional jet. **a**, The expected Doppler shifts change as the spectrometer scans across the jet structure. The vertical lines indicate the line of sight at three arbitrary scan positions of the spectrometer slit

during east-west raster. **b**, The magnetic field lines (dashed) and the plasma flow (solid arrows) for a reconnection model. The jet speed is of the order of the Alfvén speed, V_A , upstream of the current sheet. The inflow speed is much less than V_A .

are clearly displaced from each other by several arcseconds along the slit. This displacement is seen both at the jet source and at the positions on either side. In terms of the model sketched in Fig. 2a, the reversed blue and red shifts would be due to a reversal of the jet's east-west orientation with respect to the line of sight and the displacement along the slit corresponds to a marked tilt of the projected jet axis with respect to the north-south direction. Again the blue stream, indicating material flowing out of the solar surface, extends to almost 3.3 arcsec away from the source whereas the red stream, although brighter, is only seen 1.1 arcsec from the centre.

The observation that explosive events are bi-directional jets provides new evidence that they result from magnetic reconnection above the solar surface. From simultaneous magnetic field and ultraviolet measurements Dere *et al.*¹⁸ note that explosive events are often found on the chromospheric network boundary and seem to be associated with the cancellation of photospheric magnetic fields. The network consists of curtains of very strong magnetic flux tubes. All flux tubes are anchored by their footpoints to the photosphere. The continual motions in the photosphere mean that field lines of opposite polarity are naturally drawn together. If flux tubes with opposite-polarity field lines are pushed together a current sheet forms. In a finite-resistivity plasma, a small region near the neutral line may collapse and create a thin reconnection region. From this region, plasma is ejected in both directions along the field lines with a velocity of the order of the Alfvén speed^{11,12} (Fig. 2b). If there is an obstacle blocking one side, the situation is not as idealized in Fig. 2, and the flow may be uni-directional. For example, jets directed out of the Sun may stream freely up to coronal heights whereas the downward flow could quickly be slowed down by the increasing density of the chromosphere. This would in a simple manner explain the larger spatial extent of the blue-shifted wing in our observations.

The observations presented here seem to establish the association between bi-directional jets and reconnection. Since the launch of SUMER, a vast collection of data on explosive events has been accumulated. We expect that similar bi-directional jets are to be seen at and above active regions wherever reconnection occurs. The present and future observations of this kind will lead to a better understanding of how the Sun's magnetic energy feeds its hot corona and the solar wind. □

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- Giovannelli, R. G. A theory of chromospheric flares. *Nature* **158**, 81–82 (1946).
- Gold, T. & Hoyle, F. Solar flares. *Mon. Not. R. Astron. Soc.* **120**, 89–105 (1960).
- Priest, E. R. *Solar Flare Magnetohydrodynamics* (Gordon & Breach, London, 1981).
- Hundhausen, A. J. in *Proc. 6th Int. Solar Wind Conf.* (eds Pizzo, V. J., Holzer, T. E. & Sime, D. G.) 181–214 (Nat. Center for Atmospheric Res., Boulder, 1988).
- Gold, T. in *AAS-NASA Symp. on Solar Flares* (ed. Hess, W. N.) 389–395 (NASA-SP 50, NASA, Washington DC, 1964).
- Axford, W. I. & McKenzie, J. F. in *Solar Wind Seven* 1–5 (eds Marsch, E. & Schwenn, R.) (Pergamon, Oxford, 1992).
- Tsuneta, S. *et al.* Global restructuring of the coronal magnetic fields observed with the Yohkoh soft X-ray telescope. *Publ. Astron. Soc. Jpn.* **44**, L211–L214 (1992).
- Masuda, S. *et al.* A loop-top hard X-ray source in a compact solar flare as evidence for magnetic reconnection. *Nature* **371**, 495–497 (1994).
- Porter, I. G. *et al.* Microflares in the solar magnetic network. *Astrophys. J.* **323**, 380–390 (1987).
- Webb, D. F. *et al.* The correspondence between X-ray bright points and evolving magnetic features in the quiet Sun. *Solar Phys.* **144**, 15–35 (1993).
- Petschek, H. E. in *AAS-NASA Symp. on Solar Flares* (ed. Hess, W. N.) 425–437 (NASA-SP 50, NASA, Washington DC, 1964).
- Priest, E. R. *Solar Magnetohydrodynamics* (Reidel, Norwell, MA, 1982).
- Rabin, D. & Dowdy, J. F. Jr Pervasive variability in the quiet solar transition region. *Astrophys. J.* **398**, 665–681 (1992).
- Brueckner, G. E. & Bartoe, J.-D. F. Observations of high-energy jets in the corona above the quiet Sun, the heating of the corona, and the acceleration of the solar wind. *Astrophys. J.* **272**, 329–348 (1983).
- Dere, K. P., Bartoe, J.-D. F. & Brueckner, G. E. Explosive events in the solar transition zone. *Solar Phys.* **123**, 41–68 (1989).
- Dere, K. P. Explosive events, magnetic reconnection, and coronal heating. *Adv. Space Res.* **14**, 13–22 (1994).
- Brueckner, G. E. *et al.* HRTS results from Spacelab 2. *Adv. Space Res.* **6**, 263–272 (1986).
- Dere, K. P. *et al.* Explosive events and magnetic reconnection in the solar atmosphere. *J. Geophys. Res.* **96**, 9399–9407 (1991).
- Wilhelm, K. *et al.* SUMER—solar ultraviolet measurements of emitted radiation. *Solar Phys.* **162**, 189–231 (1995).
- Wilhelm, K. *et al.* First results of the SUMER telescope and spectrometer—solar ultraviolet measurements of emitted radiation—on SOHO: I. Spectra and spectrodiometry. *Solar Phys.* (in the press).

- Lemaire, P. *et al.* First results of the SUMER telescope and spectrometer—solar ultraviolet measurements of emitted radiation—on SOHO: II. Imagery and data management. *Solar Phys.* (in the press).
- Dere, K. P. *et al.* UV observations of macrospicules at the solar limb. *Solar Phys.* **119**, 55–63 (1989).

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An X-ray-induced insulator-metal transition in a magnetoresistive manganite

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Manganese oxides of the general formula $A_{1-x}B_xMnO_3$ (where A and B are trivalent and divalent cations, respectively) have recently attracted considerable attention by virtue of their unusual magnetic and electronic properties^{1–9}. For example, in some of these materials magnetic fields can drive insulator-to-metal transitions where both the conductivity and magnetization change dramatically—an effect termed ‘colossal magnetoresistance’^{1–3}—raising hopes for application of these materials in the magnetic recording industry^{1–9}. Here we show that in one such compound, $Pr_{0.7}Ca_{0.3}MnO_3$, a transition from the insulating antiferromagnetic state to the metallic ferromagnetic state can be driven by illumination with X-rays at low temperatures (<40 K). This transition is accompanied by significant changes in the lattice structure, and can be reversed by thermal cycling. This effect, undoubtedly a manifestation of the strong electron–lattice interactions believed to be responsible for the magnetoresistive properties of these materials^{8–9}, provides insights into the physical mechanisms of persistent photoconductivity, and may also find applications in X-ray detection and X-ray lithographic patterning of ferromagnetic nanostructures.

The $x = 0$ and $x = 1$ end-members of the $Pr_{1-x}Ca_xMnO_3$ family are insulating antiferromagnets with the manganese ion in the Mn^{3+} and Mn^{4+} valence states, respectively¹⁰. For intermediate x , the average Mn valence is non-integer and the material is generally semiconducting or metallic at high temperatures. At low temperatures, the charge carriers localize in a variety of structural and magnetic ordering patterns with mixed valences of the manganese ions¹⁰. The charge ordering is associated with lattice distortions which can be revealed by X-ray and neutron diffraction. In $Pr_{0.7}Ca_{0.3}MnO_3$ this metal–insulator transition occurs at temperature $T \approx 200$ K (ref. 3), and the low-temperature charge-ordering pattern^{4,10} is sketched in the inset of Fig. 1. Note that this pattern (doubling of the unit cell in one lattice direction) is commensurate with the underlying nearly-cubic lattice despite the incommensurate average Mn valence. The low-temperature magnetic structure is antiferromagnetic with a weak uncompensated moment due to spin canting⁴. The material can be driven back into the metallic state by applying a critical magnetic field H_c ; $H_c \approx 4$ T at low temperatures^{3,4}. The metallic state is ferromagnetic due to double exchange⁴. This field-induced transition is associated with large hysteresis; the material in fact remains metallic after the field is reduced to zero, but reverts to the charge-ordered state on subse-

quent heating above 60 K. Similar metastability phenomena have also been observed in other manganites⁵.

The intensity of a Bragg reflection characteristic of the low-temperature charge-ordered structure, (4, 1.5, 0), was monitored by neutron diffraction from a single-crystal sample and is shown in Fig. 1 as a function of temperature. (The reflections are indexed on an orthorhombic, though nearly cubic, lattice with lattice constants $a = 5.426 \text{ \AA}$, $b = 5.478 \text{ \AA}$ and $c/\sqrt{2} = 5.430 \text{ \AA}$; ref. 10.) The temperature dependence of the intensity at this position, where magnetic scattering contributes only very weakly, differs somewhat from that measured previously with neutrons⁴ at the (2, 1.5, 0) position where nuclear and magnetic reflections are superposed. The intensity of the equivalent (2, 1.5, 0) reflection of the same sample was also measured by X-ray diffraction (which is only sensitive to the lattice structure and not to the magnetic moments). Whereas the high-temperature ($T \geq 40 \text{ K}$) portion of the curve of Fig. 1 was reproduced with X-rays, a surprising effect occurred at lower temperatures. Figure 2 shows that the intensity of the reflection decreases continuously with X-ray illumination; no change is observed when the X-ray beam is switched off. The diminution of the charge order is associated with a dramatic change in transport properties. The conductance measured between two contacts spaced $\sim 1 \text{ mm}$ apart on a polished surface of the sample increases by more than six orders of magnitude after $\sim 20 \text{ min}$ of total X-ray exposure (Fig. 2). With the X-ray beam off, the conductivity persists for many hours without measurable degradation.

Although the metallic state generated after prolonged X-ray exposure exhibits conventional ohmic conductivity (Fig. 3b), Fig. 3a shows that the current-voltage characteristics after short exposure are remarkably nonlinear. (We have therefore quoted conductance rather than conductivity in Fig. 2). The non-ohmic conductivity in this regime is a consequence of a much more general current switching behaviour which will be discussed separately. We obtain $\rho \approx 5 \times 10^{-4} \Omega \text{ cm}$ for the ohmic resistivity after prolonged X-ray exposure, using the calculated X-ray penetration depth of $1.5 \mu\text{m}$ as the depth of the conducting channel between the contacts. This is (to within the errors) identical to the resistivity measured without X-rays above the critical magnetic field³, suggesting that the photoinduced and field-induced metallic phases are identical.

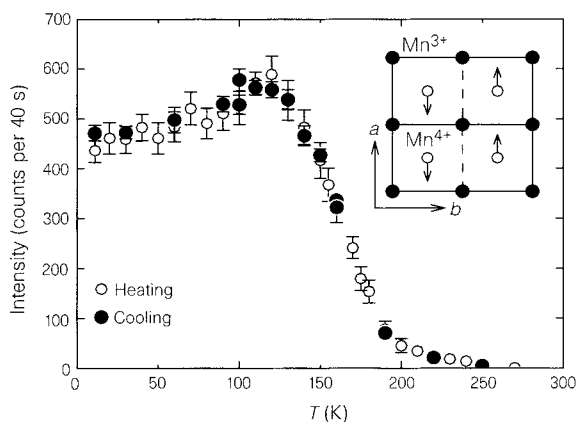


Figure 1 Intensity of a (4, 1.5, 0) superlattice reflection characteristic of the low-temperature charge ordered state of $\text{Pr}_{0.7}\text{Ca}_{0.3}\text{MnO}_3$, measured by neutron diffraction on a single-crystal sample. The crystal was grown by the floating zone technique, details of which are given in ref. 3. The measurements were taken on the H7 spectrometer at the High Flux Beam Reactor, Brookhaven National Laboratory, with 14.7-meV neutrons. The filled (open) symbols represent data taken on cooling (heating). The inset shows a two-dimensional section of the charge-ordering pattern with the primary lattice distortion.

Further strong evidence for this assertion comes from a comparison of the metastability boundaries of these two metallic states. Figure 4 shows that the X-ray-induced conductivity is annealed out on heating above 60 K, which is the annealing temperature for the magnetic-field-induced phase³. Figure 2 also shows phase coexistence of the insulating (charge-ordered) and metallic states for short exposures which may indicate that the photoinduced transition is first order, as is the field-induced transition³. We have also repeated our X-ray measurements in magnetic fields up to H_c and find that the photoinduced transition occurs faster under these conditions. Though the magnetization was not measured directly, the close analogy to the magnetic-field-induced transition implies that the magnetic properties of the sample change dramatically with illumination, from canted antiferromagnetic to ferromagnetic. To our knowledge, a photoinduced antiferromagnetic-to-ferromagnetic transition has thus far not been observed.

We have considered several possible mechanisms for the observed photoinduced transition. First, photoinduced oxygen diffusion has been implicated in photoconductivity in some layered and amorphous oxides with very large oxygen mobilities^{11,12}, but cannot play a role in this cubic material. An oxygen-diffusion mechanism is also ruled out by the above comparison of photoinduced and magnetic-field-induced transitions. Further, beam heating cannot be responsible because the conductivity and the Bragg peak intensity do not recover when the beam is switched off. We thus conclude that the increased conductivity is caused by X-ray photoelectrons and secondary electrons generated in collisions.

Significant transient X-ray photocurrents have been observed in a variety of materials such as amorphous Se (ref. 13), but to our knowledge persistent X-ray photoconductivity has not yet been reported. To explain why the X-ray-generated carriers are not recaptured when the beam is switched off, it helps to recall models for persistent (visible-light) photoconductivity in III-V semiconductors in the presence of DX centres^{14,15}. These centres are thought to be associated with large lattice distortions which can be thermally excited at high temperatures, resulting in trapping of charge carriers. When carriers are photoexcited out of the traps at low temperatures, however, the lattice relaxes and the capture cross-section (mediated only by zero-point fluctuations of the lattice) becomes extremely small. Despite the different photon energies

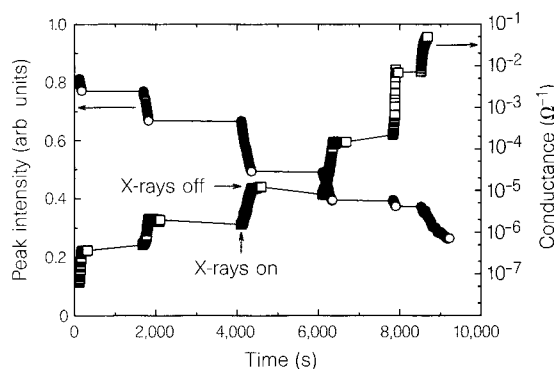


Figure 2 X-ray exposure dependence of the peak intensity of the (2, 1.5, 0) Bragg reflection characteristic of charge ordering (left axis), and of the electrical conductance (right axis), at temperature $T = 4 \text{ K}$. The measurements were taken with a monochromatic X-ray beam (energy 8 keV, flux $5 \times 10^{10} \text{ s}^{-1}$, beam size $1 \times 1 \text{ mm}$) at beamline X22B, National Synchrotron Light Source.

used in the experiments, the phenomenology of DX centres, including in particular the annealing of the metastable conducting state at elevated temperatures, bears a striking resemblance to our observations in $\text{Pr}_{0.7}\text{Ca}_{0.3}\text{MnO}_3$.

These observations have important implications both for the mechanism of colossal magnetoresistance (CMR) and for the physics of persistent photoconductivity in general. One of the most perplexing aspects of the physical properties of the manganites is their insulating state; traditional models of these materials cannot account

for this⁶. Current theories of CMR⁶ therefore invoke strong polaronic self-trapping of the carriers mediated by the Jahn–Teller distortion of the Mn^{3+} ion. In conjunction with the DX centre analogy, our data clearly illustrate the dominant role of electron–lattice coupling as a driving force for charge localization and suggest that the insulating state in $\text{Pr}_{0.7}\text{Ca}_{0.3}\text{MnO}_3$ should be regarded as a polaron lattice. Conversely, the lattice distortions around DX centres have thus far not been observed directly, which leaves a gap in an otherwise reasonably complete understanding of persistent photoconductivity. This gap is filled by the diffraction data of Fig. 2 which demonstrate unambiguously that the lattice relaxes when photoelectrons are removed from the charge-ordered state of $\text{Pr}_{0.7}\text{Ca}_{0.3}\text{MnO}_3$. The large relaxation of the lattice on entering the same metallic state through two different routes (X-rays and magnetic field) also provides a common microscopic explanation of both the newly discovered persistent photoconductivity and the irreversibility effects observed in previous magnetotransport experiments on $\text{Pr}_{0.7}\text{Ca}_{0.3}\text{MnO}_3$ and other manganites^{3,5}.

The initial experiments reported here open the way for a variety of further experimental studies. For instance, further insight into the mechanism of the photoinduced transition could be gained by studying its dependence on photon energy. Preliminary experiments show that the kinetics of the transition are not strongly affected when the X-ray energy is lowered below the Mn K absorption edge, which may indicate that these phenomena occur over a wide energy range. From the point of view of applications, the unique properties of the manganites may, with further development, prove useful in X-ray lithography and X-ray detection. Perhaps even more importantly, the evidence presented here strongly suggests that the photoinduced phase is not only metallic but also ferromagnetic. Using X-ray lithography it should thus be feasible to pattern very small ferromagnetic structures into these materials, which would open up new possibilities for both fundamental and applied research on magnetism. In a compound of slightly modified composition, $\text{Pr}_{0.65}\text{Ca}_{0.245}\text{Sr}_{0.105}\text{MnO}_3$, we have recently observed significant persistent X-ray photoconductivity at temperatures in excess of 100 K, an important step towards practicability of these ideas. □

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- Chahara, S., Ohno, T., Kasai, K. & Kozono, Y. Magnetoresistance in magnetic manganese oxide with intrinsic antiferromagnetic spin structure. *Appl. Phys. Lett.* **63**, 1990–1992 (1993).
- Im, S. *et al.* Thousandfold change in resistivity in magnetoresistive La–Ca–Mn–O films. *Science* **264**, 413–415 (1994).
- Tomiooka, Y., Asamitsu, A., Kuwahara, H., Moritomo, Y. & Tokura, Y. Magnetic-field-induced metal-insulator phenomena in $\text{Pr}_{1-x}\text{Ca}_x\text{MnO}_3$ with controlled charge-ordering instability. *Phys. Rev. B* **53**, R1689–R1692 (1996).
- Yoshizawa, H., Kawano, H., Tomiooka, Y. & Tokura, Y. Neutron-diffraction study of the magnetic-field-induced metal-insulator transition in $\text{Pr}_{1-x}\text{Ca}_x\text{MnO}_3$. *Phys. Rev. B* **52**, R13145–R13148 (1995).
- Tokura, Y., Kuwahara, H., Moritomo, Y., Tomiooka, Y. & Asamitsu, A. Competing instabilities and metastable states in $(\text{Nd}, \text{Sm})_{1-x}\text{Sr}_x\text{MnO}_3$. *Phys. Rev. Lett.* **76**, 3184–3187 (1996).
- Millis, A. J., Littlewood, P. M. & Shraiman, B. I. Double exchange alone does not explain the resistivity of $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$. *Phys. Rev. Lett.* **74**, 5144–5147 (1995).
- Park, J. H. *et al.* Electronic aspects of the ferromagnetic transition in manganese perovskites. *Phys. Rev. Lett.* **76**, 4215–4218 (1996).
- Billinge, S. J. L., DiFrancesco, R. G., Kwei, G. H., Neumeier, J. J. & Thomson, J. D. Direct observation of lattice polaron formation in the local structure $\text{La}_{1-x}\text{Ca}_x\text{MnO}_3$. *Phys. Rev. Lett.* **77**, 715–718 (1996).
- Shenglaya, A., Zhao, G. M., Keller, H. & Müller, K. A. EPR evidence of Jahn–Teller polaron formation in $\text{La}_{1-x}\text{Ca}_x\text{MnO}_3$. *Phys. Rev. Lett.* **77**, 5296–5299 (1996).
- Ito, K., Krupicka, S., Simsa, Z., Dlouha, M. & Vratislav, S. Neutron diffraction study of $\text{Pr}_{1-x}\text{Ca}_x\text{MnO}_3$ perovskites. *J. Magn. Magn. Mat.* **53**, 153–166 (1985).
- Pashmakov, B., Claflin, B. & Fritzsche, H. Photoreduction and oxidation of amorphous indium oxide. *Solid State Commun.* **86**, 619–622 (1993).
- Kudinov, V. I. *et al.* Persistent photoconductivity in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ films as a method of photodoping toward metallic and superconducting phases. *Phys. Rev. B* **47**, 9017–9028 (1993).
- Donovan, J. L. X-ray sensitivity of selenium. *J. Appl. Phys.* **50**, 6500–6554 (1979).
- Mooney, P. M. Deep donor levels (DX centers) in III–V semiconductors. *J. Appl. Phys.* **67**, R1–R26 (1990).
- Lang, D. V. in *Deep Centres in Semiconductors* (ed. Pantelides, S. T.) 489–539 (Gordon & Breach, New York, 1986).

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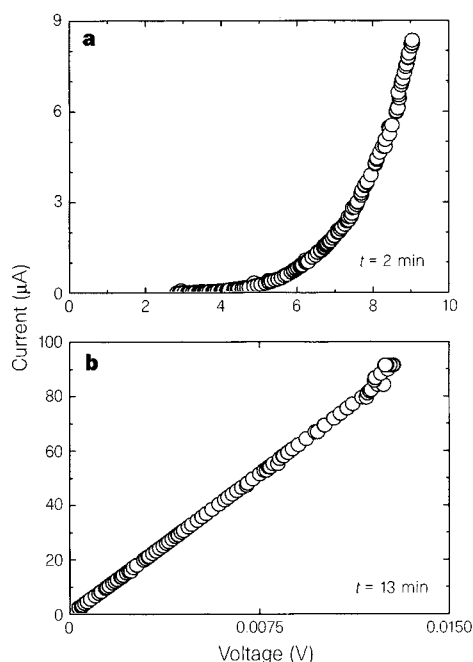


Figure 3 Current–voltage characteristics measured after different X-ray exposures at temperature $T = 4$ K.

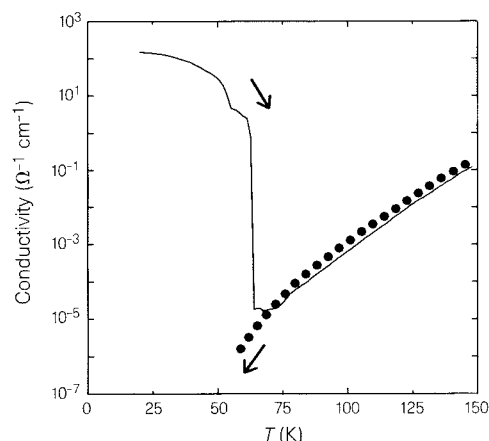


Figure 4 Conductivity measured on cooling before X-ray illumination (dotted curve) and on heating after illumination with X-rays for a moderate amount of time (solid curve). In the unilluminated state, the conductivity was measured directly and is consistent with the measurements of ref. 3. X-ray illumination creates a conducting channel of depth equal to the X-ray penetration depth ($\sim 1.5 \mu\text{m}$) between two contacts spaced ~ 1 mm apart on a polished surface of the sample. Conductance data taken in the illuminated, unannealed state at low temperatures were converted to conductivity by using the calculated channel depth.

Fingering in granular flows

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The flow of granular materials on inclined planes is of interest within the contexts of both industrial processing of powders and geophysical instabilities such as landslides and avalanches^{1,2}. These flows have been found to be complex, exhibiting several different flow regimes^{3–7} as well as particle segregation effects^{8,9} and instabilities¹⁰. Here we describe an instability that occurs when a front of granular material propagates down a rough inclined plane. The front, which is initially uniform in cross-section, rapidly breaks up into fingers. Although this is similar in appearance to the instability seen in viscous fluids flowing down a plane^{11–17}, in these latter cases the instability is driven by surface tension, whereas granular materials have no surface tension. We show that the fingering instability in this case is instead induced by the size segregation that develops during the flow.

The experimental set-up is shown in Fig. 1. A 2-m-long and 70-cm-wide glass surface is roughened by gluing onto its surface a single layer of 0.5-mm-diameter glass beads. The rough bed is transparent and allows the system to be viewed from below. A double gate system (Fig. 1a) can be opened suddenly to give an opening h_g constant across the bed. When the inclination angle α is sufficiently large, the material pours through the gate, forming a well defined front propagating down the slope.

The preliminary experiments were carried out using poor-quality glass beads 0.5 mm in mean diameter (material no. 1). By 'poor quality' we mean that the glass beads were not properly sieved and

contained some clusters of beads bonded together forming large and irregular particles. With this material the propagation of the front exhibits an instability as shown in Fig. 1b: the front rapidly breaks up into fingers. This instability is observed for a wide range of inclination angle α between 26° and 34° and for different gate openings h_g to a maximum of 16 particle diameters. For higher inclinations or deeper layers, the flow is continuously accelerating or leads to other free-surface instabilities.

A first step in the understanding of the origin of the fingering was obtained from experiments carried out with a second set of beads (material no. 2) which were quasi-monodispersed spherical glass beads sieved between 0.45 and 0.56 mm. With this medium no fingering was observed, and the front remained uniform, regardless of the inclination or the gate opening.

This surprising result indicates that the polydispersity of the granular medium plays an important role in the instability. More precisely, we have found that a necessary condition for the occurrence of the fingering is the presence of coarse irregular particles in the material. The addition to the spherical glass beads of 5% by volume of larger irregular-shaped particles (crushed fruit stones, material no. 3) sieved between 0.50 and 0.63 mm is sufficient to induce the development of fingers. In contrast, the addition of small irregular particles sieved under 0.40 mm leads to a stable front. The fingering is thus entirely driven by the motion of the coarse irregular particles present in the material, suggesting that the segregation occurring during the flow plays a crucial role.

It is well known that, in inclined chute flows of polydispersed media, the coarse particles come to the free surface^{7–9}. This phenomenon can be explained by a statistical sieving mechanism^{8,9}. In the case of propagating fronts, the vertical segregation occurring far from the front gives rise to a complex recirculation zone^{7,10,18} at the front; in our experiments, this recirculation turns out to be the origin of the fingering instability.

We have studied this recirculation zone for materials made of a mixture of the spherical glass beads (material no. 2) and the crushed

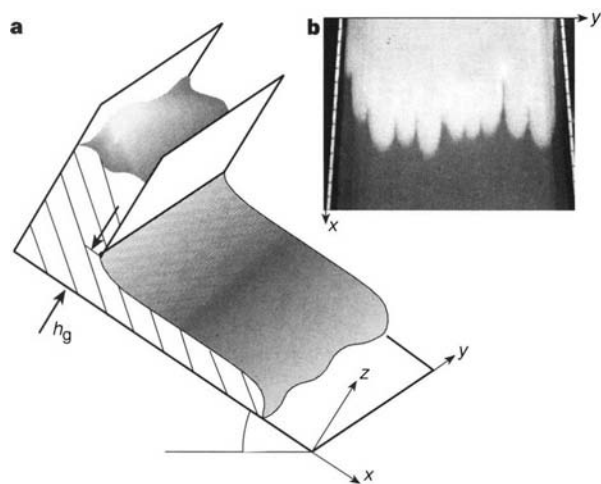


Figure 1 a, Experimental set-up. Three different granular materials were used. Material no. 1: poor-quality glass beads, mean diameter $d = 0.5$ mm; the mean density in close packing was $\rho = 1.5$ g cm⁻³ and the bed friction angle was $\delta = 26^\circ$ (δ here is the critical angle at which a 10-diameter-thick layer flows). Material no. 2: quasi-monodispersed spherical glass beads $0.45 < d < 0.56$ mm, $\rho = 1.6$ g cm⁻³, $\delta = 22^\circ$. Material no. 3: vegetal abrasive (crushed fruit stones) $0.5 < d < 0.63$ mm, $\rho = 0.7$ g cm⁻³, $\delta = 33^\circ$. **b**, Deformation of the front observed for the poor-quality glass beads (material no. 1), $\alpha = 30.5^\circ$, $h_g = 6$ mm.

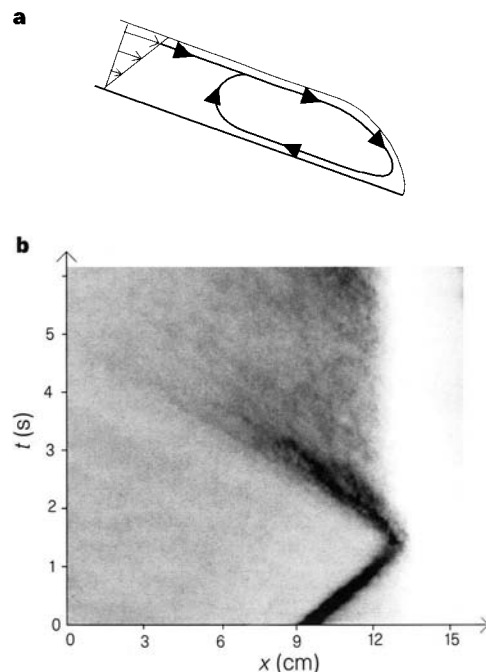


Figure 2 a, Sketch of the recirculation of the large particles at the front. **b**, Spatio-temporal evolution of a line of black tracers (material no. 3) released on a flow of glass beads (material no. 2); $\alpha = 24^\circ$, $h_g = 4$ mm.

fruit stones (material no. 3). The motion of the large particles that we observed in our experiments is sketched in Fig. 2a. At the outlet of the reservoir the large particles rapidly segregate and arrive at the front flowing on the free surface where the velocity is higher. They reach the front and stop on the bed, while the front continues to propagate down the slope. The large particles are thus re-injected in the material (Fig. 2a). The particles then rise up again to the free surface as the segregation process takes place, giving rise to a recirculation motion in a frame moving with the front. Experimental evidence of this phenomena is given in Fig. 2b which represents the spatiotemporal evolution of a line of large black particles of material no. 3 released at the free surface of a flow of pure glass beads. Pictures were taken by a CCD (charge-coupled device) camera placed below the bed and moving with the mean front velocity. The glass beads being translucent, the grey level on the pictures is approximately proportional to the mean concentration of tracers integrated across the avalanching layer. Figure 2b thus gives the time evolution of the tracers' concentration profile close to the front. The black tracers are released 4 cm upstream from the front at $t = 0$ s. They reach the front for the first time at $t = 1.3$ s and then move backward (as sketched in Fig. 2a) before rising up to the free surface; this gives the V-shape on the spatiotemporal diagram. The next loop followed by the tracers is more difficult to observe: the recirculation length appears to vary considerably from one particle to another (over the range 1–12 cm) and also for a given particle from one loop to another, leading to a rapid enlargement of the tracer strip. However, the large particles stay in the vicinity of the front as shown by the enhancement of the grey level close to the front in Fig. 2b for $t > 3$ s. The details of this recirculation process will not be examined here. Rather, we focus on the mechanism of the fingering instability resulting from this basic motion of the large irregular particles.

The initiation of the instability is sketched in Fig. 3a and is entirely driven by the path of the large particles which arrive at the front line on the free surface (black arrows) and leave it at the bed

(white arrows). If a small perturbation occurs at the front, the trajectories of the large particles arriving at the front are deflected towards the dip of the deformation, following the steepest slope of the free surface. This is clearly observed in Fig. 3b which is the average of 28 pictures taken every 0.02 s. However, the return trajectories of the coarse particles when they have just left the front remain approximately straight lines: they are close to the bed where the velocity in the laboratory frame is zero (this picture is correct only close to the front where the large particles have not yet moved up to the free surface). A uniform concentration of large particles arriving at the free surface thus leads to a non-uniform distribution at the bed with high concentration at the dip of the deformed front (Fig. 3a and c). This local increase of the concentration of large particles, together with the fact that they are irregular particles having a larger coefficient of friction, leads to a local increase of the friction. The material thus locally slows down which promotes amplification of the deformation which ultimately leads to the formation of fingers. The recirculation motion of the large particles amplifies this phenomenon. The accumulation of the large particles in lines resulting from the mechanism proposed in Fig. 3a is observed at the beginning of front deformation, as shown in Fig. 3c.

This distribution in strips perpendicular to the mean motion is reminiscent of the axial segregation observed in rotating drums (ref. 19 and references therein). However, the basic flows in both configurations are so different that no *a priori* analogy could be suggested. The fingering observed here however, could be related to the pattern formation observed in a rotating drum by Caponeri *et al.*²⁰. The connection between those two phenomena and the role of the fingering for the mixing properties in rotating drums represent interesting topics for future investigation. Another interesting topic in a geophysical context would be to study whether this segregation-induced fingering mechanism is relevant for real landslides, which sometimes exhibit finger-like patterns. □

Received 14 November 1996; accepted 10 March 1997.

1. Savage, S. B. & Hutter, K. The motion of a finite mass of granular materials down a rough incline. *J. Fluid Mech.* **199**, 177–215 (1989).
2. Savage, S. B. & Hutter, K. Dynamics of avalanches of granular materials from initiation to runout. Part I: Analysis. *Acta Mech.* **86**, 201–223 (1991).
3. Augenstein, D. A. & Hogg, R. An experimental study of the flow of dry powders over inclined surfaces. *Powder Technol.* **19**, 205–215 (1978).
4. Savage, S. B. Gravity flow of cohesionless granular materials in chutes and channels. *J. Fluid Mech.* **92**, 53–96 (1979).
5. Drake, T. J. Structural features in granular flows. *J. Geophys. Res.* **95**, 8681–8696 (1990).
6. Anderson, K. G. & Jackson, R. A comparison of the solutions of some proposed equations of motion of granular materials for fully developed flow down inclined planes. *J. Fluid Mech.* **92**, 145–168 (1992).
7. Vallance, J. W. Experimental and field studies related to the behavior of granular mass flows and the characteristics of their deposits. Thesis, Michigan Technol. Univ. (1994).
8. Drahn, J. A. & Bridgwater, I. The mechanisms of free surface segregation. *Powder Technol.* **36**, 39–53 (1983).
9. Savage, S. B. & Lun, C. K. Particle size segregation in inclined chute flow of dry cohesionless granular solids. *J. Fluid Mech.* **189**, 311–335 (1988).
10. Davies, R. H. Debris-flow surges—Experimental simulation. *J. Hydrol. (NZ)* **29**, 18–46 (1990).
11. Huppert, H. E. Flow and instability of a viscous current down a slope. *Nature* **300**, 427–429 (1982).
12. Trojan, S. M., Herbolzheimer, E., Safran, S. A. & Joanny, J. F. Fingering instabilities of driven spreading films. *Europhys. Lett.* **10**, 25–30 (1989).
13. de Bruyn, J. R. Growth of fingers at a driven three-phase contact line. *Phys. Rev. A* **46**, 4500–4503 (1992).
14. Brenner, M. P. Instability mechanism at driven contact lines. *Phys. Rev. E* **47**, 4597–4599 (1993).
15. Spaid, M. A. & Homsy, G. M. Stability of Newtonian and viscoelastic dynamic contact lines. *Phys. Fluids* **8**, 460–478 (1996).
16. Melo, F., Joanny, J. F. & Fauve, S. Fingering instability of spinning drops. *Phys. Rev. Lett.* **63**, 1958–1961 (1989).
17. Frayssé, N. & Homsy, G. M. An experimental study of rivulet instabilities in centrifugal spin coating of viscous newtonian and non-newtonian fluids. *Phys. Fluids* **6**, 1491–1504 (1994).
18. Savage, S. B. in *Theoretical and Applied Mechanics* (eds Germain, P., Piau, M. & Callerie, D.) 241–266 (Elsevier, Amsterdam, 1989).
19. Zik, O., Levine, D., Lipson, S. G., Shtrikman, S. & Stavans, J. Rotationally induced segregation of granular materials. *Phys. Rev. Lett.* **73**, 644–647 (1994).
20. Caponeri, M., Douady, S., Fauve, S. & Laroche, C. in *Mobile Particulate Systems* (eds Guazzelli, E. & Oger, L.) 619 (NATO ASI Ser. E Vol. 11, Kluwer Academic, The Netherlands, 1995).

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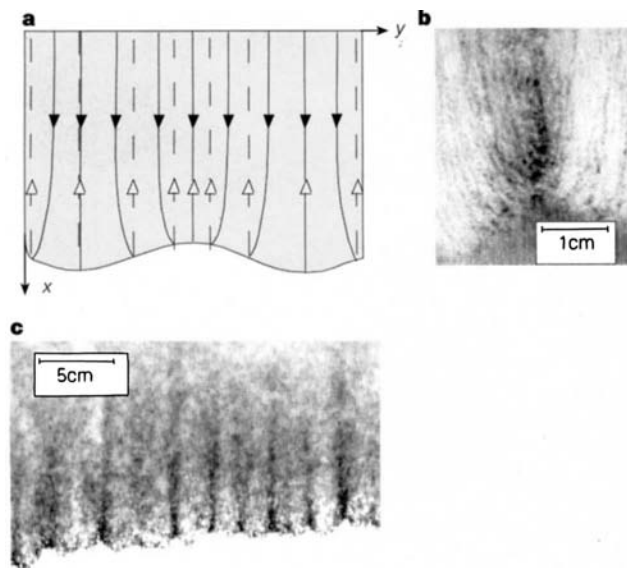


Figure 3 **a**, Instability mechanism; the black (white) arrows represent the trajectories of the coarse particles on the top (bottom) of the avalanching material. **b**, Averaged picture of 28 top views of the front taken every 0.02 s; **c**, bottom view of the front. The mixture is comprised of 5 vol.% material no. 3 and 95 vol.% material no. 2; $\alpha = 25^\circ$, $h_0 = 5$ mm.

Rapid climate change in the North Atlantic during the Younger Dryas recorded by deep-sea corals

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Research on global climate change has increasingly focused on rapid (century-scale and decadal) changes. One such climate shift, the Younger Dryas cooling event¹, took place during the last deglaciation, from 13,000 to 11,700 years BP. Climate records from Greenland ice cores and North Atlantic sediment cores show high-frequency fluctuations implying significant (>5 °C) shifts in temperature at this time, taking place within 50–100 years (ref. 2). The origin of the Younger Dryas has recently been attributed to a reduction or cessation of deep-water production in the North Atlantic and a concurrent lessening of the heat flux from low latitudes^{3,4}. The role of intermediate waters (1,000–2,000 m depth) is less certain, however, because climate proxies for this ocean reservoir are rare and ambiguous. Here we report on the use of a new climate archive, deep-sea corals from Orphan knoll (1,600 m depth) in the northwestern Atlantic Ocean. The oxygen isotope ratios in the coral skeletons (accurately dated by the ²³⁰Th/²³⁴U chronometric method) change markedly coincident with the initiation of the Younger Dryas, suggesting that there were profound changes in intermediate-water circulation at this time.

Evidence concerning past oceanographic conditions is derived largely from isotopic analyses of biogenic carbonates. Temperature affects the partitioning of oxygen isotopes between sea water and precipitating carbonates, while ocean circulation patterns and other climatically important factors affect both the oxygen and carbon isotopic compositions of sea water. Carbonates from foraminifera and reef corals have been especially useful sources of isotopic data; foraminifera are, however, difficult to date accurately and reef corals only live in shallow tropical waters. Non-reef-building, non-photosynthetic corals potentially offer the advantages of both groups: widespread distribution (from surface to abyssal depths, and from polar to equatorial latitudes⁵), suitability for ²³⁰Th/²³⁴U disequilibrium dating, and more-or-less continuous skeletal deposition over periods of decades to centuries.

Attempts to use deep-sea corals as recorders of ambient oceanographic conditions have so far yielded ambiguous results^{6–8}. The main problem is that the skeletons of deep-sea corals usually do not precipitate in isotopic equilibrium with sea water, and the extent of the disequilibrium varies widely within a particular individual. Multiple isotopic analyses of individual modern deep-sea corals collected live from waters of known temperature and isotopic composition show strong positive $\delta^{18}\text{O}/\delta^{13}\text{C}$ correlations⁹. Regression lines extend from a point at or near ¹⁸O equilibrium with sea water but a few per mil depleted (with respect to equilibrium) in ¹³C, to a point up to 4‰ depleted in ¹⁸O and up to 12‰ depleted in ¹³C. These features have been explained as a superposition of 'kinetic' and 'metabolic' isotope effects¹⁰. Slower CO₂ hydration and hydroxylation reactions by molecules bearing the heavy isotopes ¹³C and ¹⁸O cause the kinetic effects. Partial re-equilibration accounts for the strong positive correlations between skeletal $\delta^{18}\text{O}$

and $\delta^{13}\text{C}$. Skeletal incorporation of ¹³C-depleted respired CO₂ causes the metabolic effects: these effects are probably small in deep-sea corals, however, as respired CO₂ contributes little to their skeletal makeup¹¹.

Even though considerable isotopic disequilibria were observed, the regression lines for the data from individual corals (plotted as $\delta^{18}\text{O}$ versus $\delta^{13}\text{C}$) were offset from each other as a function of temperature⁹. In each case, the heaviest isotopic ratios approach or reach oxygen equilibrium with sea water; the carbon-isotope data were always depleted with respect to equilibrium by varying

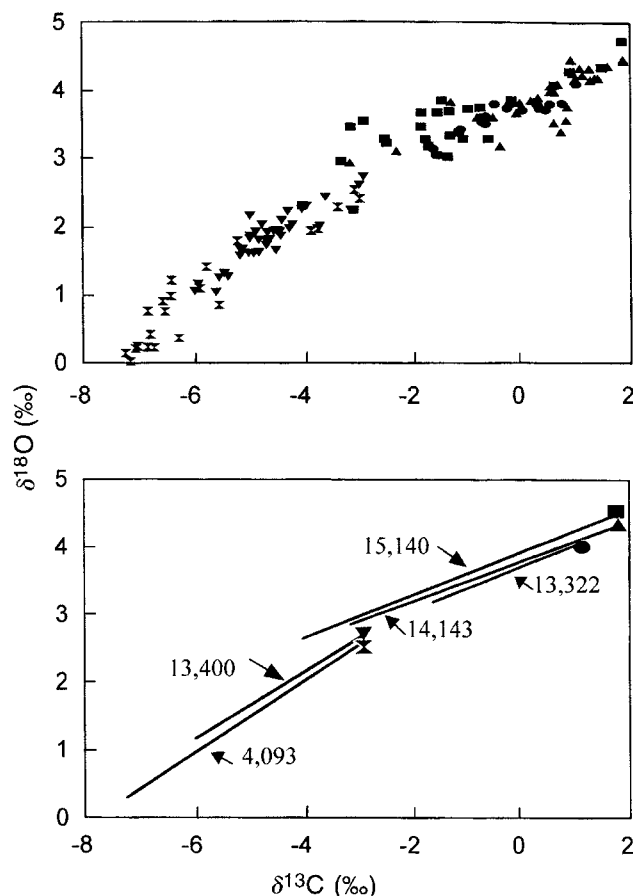


Figure 1 Top, scatter plot of $\delta^{18}\text{O}$ versus $\delta^{13}\text{C}$ values for four Orphan knoll corals. Filled squares, isotopic values from coral no. 14, dated at 15,140 ± 104 absolute (calendar) years BP; upright triangles (▲), data from coral no. 23 at 14,143 ± 86 yr BP; upside-down triangles (▼), data from the lower part of coral no. 1 from skeleton growing at ~13,400 yr BP; circles (●), data from subsamples taken from the upper part of coral no. 1, dated at 13,322 ± 98 yr BP. Hourglass symbols, isotopic values from a submodern specimen (coral no. 27 at 4,093 ± 44 yr BP). Note that there is no relationship between the position of an individual datum on the plot and its physical location on the corals: its position is a function of temperature, isotopic composition of the sea water and magnitude of kinetic isotope effect. Bottom, regression lines for data from each coral (numbers 14, 23 and 27) and the two sections, lower (earlier) and upper (later), of coral no. 1. Ages of each individual coral are given beside the regression lines. The two glacial-aged corals (numbers 14 and 23) closely resemble each other and the Younger Dryas-aged coral (the upper portion of coral no. 1). The coral skeleton growing just before the onset of the Younger Dryas (the lower portion of coral no. 1) is similar to the submodern specimen (no. 27). Note that the regression lines indicating analyses from a single specimen (coral no. 1) show that the shift from one data set to the other occurred between subsamples spaced <3 mm apart. Symbols at the right-hand end of each regression line indicate the maximum $\delta^{18}\text{O}$ value for each coral: these values approach (or reach) oxygen equilibrium with sea water; some of these values are displayed in Table 1.

amounts. The heaviest $\delta^{18}\text{O}$ values for each coral were plotted against temperature, resulting in a trend parallel to the biogenic aragonite equilibrium curve¹². This demonstrates that, although the isotopic signals from each individual coral represent a superposition of kinetic components on equilibrium values, the environmentally controlled portion (reflecting the ambient temperature and isotopic composition of the water) should be extractable from fossil corals, and may be used to reconstruct palaeoceanographic conditions.

In 1978, the Atlantic Geoscience Centre (Geological Survey of Canada) recovered dredge samples from the top of Orphan knoll, at the edge of the continental rise 550 km northeast of Newfoundland (50° 25.57' N, 46° 22.05' W)¹³. Several well preserved, dead specimens of *Desmophyllum cristagalli* Milne Edwards and Haime, 1848, were recovered. The corals were stored for several years at the Atlantic Geoscience Centre; unfortunately, much of the collection was discarded because of space problems. This robust species of deep-sea coral¹⁴ attaches to suitable hard substrates, often vertical to sub-vertical rock walls. Several of the fossil Orphan knoll specimens were dated at McMaster and at Lamont-Doherty by the uranium-series method using thermal ionization mass spectrometry (TIMS) and were found to range in age from 77 to 4 kyr (ref. 15). We chose to use uranium-series rather than radiocarbon dating, because $^{14}\text{C}/^{12}\text{C}$ ratios in fossil deep-sea corals may be influenced not only by radioactive decay and atmospheric variations, but also by the ventilation history of the deep ocean¹⁶. The calculated initial $^{238}\text{U}/^{234}\text{U}$ ratios in the coral skeletons were only slightly less than the seawater activity ratio, indicating a closed system. As the amount of ^{232}Th in the corals was negligible, a detrital thorium correction was not required. Various parts of the coral were isotopically analysed, but the data presented here derive only from the outer theca of the pedicels from above the base to below the calices. These parts of the skeleton displayed the strongest linear correlations between $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$.

Among the dated specimens, four corals were of particular interest because their dates spanned much of the last deglaciation. The stable-isotope data are shown in Fig. 1; each set of isotopic ratios and matched regression line represents the data retrieved from one dated specimen. Dates are given with 2σ uncertainties. The oldest coral discussed here (coral no. 14 at $15,140 \pm 104$ yr BP) grew during the middle of the Bølling chronozone, when the North Atlantic Ocean still retained much of its glacial character. The heaviest $\delta^{18}\text{O}$ value of the set, which presumably approaches the oxygen-equilibrium value, is 4.51‰ . By $14,143 \pm 86$ yr BP (coral no. 23), the highest skeletal value had dropped to 4.34‰ , presumably reflecting climate warming during deglaciation. The base of coral no. 1 was dated at $13,636 \pm 103$ yr BP, and the top at $13,322 \pm 98$ yr BP: from the base to about two-thirds the way up coral no. 1 (to approximately the 13,400 yr BP mark) the highest skeletal oxygen-isotope value was 2.73‰ . This total drop of 1.78‰ comprises signals both from the warming of the ambient sea water plus changes in the $\delta^{18}\text{O}$ of the sea water attributable to the addition of melt water to the North Atlantic. Following this mid-Allerød low, the $\delta^{18}\text{O}$ versus $\delta^{13}\text{C}$ plot for the same specimen, coral no. 1, changed abruptly, from one sampling location on the same coral

to the next 3 mm away, to give a non-overlapping data set with a significantly different slope (t -test, $p < 0.01$) dated at $13,322 \pm 98$ yr BP. Its highest $\delta^{18}\text{O}$ value is 4.05‰ , implying a sudden return to glacial-like conditions at this intermediate-depth site. We interpret this shift, an increase of 1.32‰ , as representing the initiation of the Younger Dryas event, which has been widely documented in ice cores, ocean sediment cores, terrestrial pollen, insect records and other climate proxies from various parts of the world, including the circum-Atlantic region^{17–19}.

None of the other deep-sea corals analysed, including corals of similar size but with ages lying outside the Younger Dryas, show such abrupt shifts in $\delta^{18}\text{O}$ ratios. Equally important, the isotopic shift at the apparent onset of the Younger Dryas occurred between two seemingly identical subsamples within 3 mm of skeletal growth. There is no evidence of any growth hiatus, or change in texture or morphology of the coral at the transition. If we assume that this fossil coral had growth rates comparable to those of modern representatives and that the $^{230}\text{Th}/^{234}\text{U}$ dates are accurate, the abrupt shift in the isotopic values along coral no. 1 suggests that the initiation of the Younger Dryas may have taken place over as few as 5 years. This rate of change in deeper-ocean conditions is one of the most rapid such recorded events in the marine record.

The isotopic record of the youngest coral (coral no. 27 at $4,093 \pm 44$ yr BP), with a maximum $\delta^{18}\text{O}$ value of 2.54‰ , reflects modern interglacial ocean conditions and is only slightly lower than $\delta^{18}\text{O}$ of the base of coral no. 1. As with the suite of modern corals, the changes in $\delta^{13}\text{C}$ are difficult to explain; clearly, a 5‰ shift in the carbon-isotope composition of sea water is improbable. This is an area in which further research is required.

North Atlantic Deep Water (NADW) production releases approximately 1 petawatt of heat ($1 \text{ PW} = 10^{15} \text{ J s}^{-1}$) to the atmosphere²⁰. A shutdown of this overturning cell (the so-called heat 'conveyor') has been implicated in past studies of the Last Glacial Maximum (LGM) and Younger Dryas climate intervals^{3,4}. Recent studies^{21–23}, however, have shown that although the deepest portions of the North Atlantic did not seem to 'feel' the presence of NADW during the LGM and the Younger Dryas, other proxies imply that the conveyor was still in operation. It has been suggested that the conveyor shoaled, that is, decreased the penetration depth of NADW, rather than shut down. Intermediate waters were then formed at the expense of the densest components of the NADW. If this is true, the mid-depth water should show distinct changes in temperature and isotopic composition as the North Atlantic shifted from one circulation mode to another. We interpret our data as lending support to this hypothesis: the two glacial-aged corals show that the ambient water had significantly different characteristics than the mid-depth waters of early deglaciation. The coral that grew during that time (13.6–13.4 kyr BP) recorded similar conditions to those experienced by the submodern (4.1 kyr BP) specimen. During the Younger Dryas, however, the intermediate waters appear to resemble closely those of the last glacial period and imply a similar provenance.

Records of changes in the isotopic composition of benthic foraminifera from mid-depth North Atlantic sites^{24,25} are virtually identical in magnitude to our isotopic results for the deep-sea corals (Table 1). The $\delta^{18}\text{O}$ record from the Orphan knoll corals, however, is a much more accurately and precisely dated record of the last deglaciation. Average 2σ uncertainty of the seven $^{230}\text{Th}/^{234}\text{U}$ dates is 87 years, whereas bioturbation, uncertainties in the radiocarbon calibration and in the ^{14}C reservoir age can bring the typical age uncertainty in foraminiferal or other microfaunal analyses to thousands of years²⁶. Errors in the ice-core record²⁷ are also too large to determine accurately the phase relationship between ice-core accumulation or $\delta^{18}\text{O}$ stratigraphy and the Orphan knoll $\delta^{18}\text{O}$ record. Accurate and precise dating is crucial in the investigation of chronozones as condensed as the Younger Dryas event, as subtle shifts in age assignments can significantly alter the interpretation of deep-ocean responses to climate change²⁸. One record with comparable

Table 1 Comparisons of available intermediate-depth foraminiferal isotopic values

	Oppo and Lehman ²⁵	Labeyrie <i>et al.</i> ²⁴	This study
Glacial	3.77–4.60‰	4.1–4.8‰	4.51‰
Younger Dryas		3.1–3.9‰	4.05‰
Modern	2.29–2.77‰	2.0–3.0‰	2.54‰
$\Delta\delta^{18}$ (Glacial–YD)		0.2–1.7‰	0.46‰
$\Delta\delta^{18}$ (Glacial–Modern)	1.0–1.31‰	1.1–2.8‰	1.97‰

The values shown are for intermediate depths (1,000–2,000 m).

accuracy to ours is the $^{230}\text{Th}/^{234}\text{U}$ -dated Barbados sea-level record²⁸. Using this, along with data from other drowned reefs in the Caribbean–Atlantic province, Blanchon and Shaw²⁹ suggested that ocean–atmosphere reorganization resulted from atmospheric changes induced by ice-sheet collapse, which in turn led to a series of catastrophic sea-level rises. One of our deep-sea corals ($14,143 \pm 104 \text{ yr BP}$) originates at (or very near) the time of one of these proposed sea-level rises ($14.2 \pm 0.1 \text{ yr BP}$), but shows no evidence of a rapid oceanographic change. The change in ocean circulation suggested by the Orphan knoll data at $\sim 13.3 \text{ kyr BP}$ is not associated with a sea-level rise, lending support to Fairbanks' assertion²⁸ that the Younger Dryas occurred at a time of minimum ice-sheet melting.

Shallow-water corals are recognized as environmental recorders without equal³⁰. Deep-water corals afford us the opportunity to access previously unavailable records of climate change. Study of deep-water corals may well lead to a new temperature archive, a more complete understanding of past temperature changes and better prediction of future changes. □

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1. Fairbanks, R. G. The age and origin of the Younger Dryas event in Greenland ice cores. *Paleoceanography* **5**, 937–948 (1990).
2. Johnsen, S. J. et al. Irregular glacial interstadials recorded in a new Greenland ice core. *Nature* **359**, 311–313 (1992).
3. Broecker, W. S. & Denton, G. H. The role of ocean–atmosphere reorganization in glacial cycles. *Geochim. Cosmochim. Acta* **53**, 2465–2501 (1989).
4. Broecker, W. S., Peteet, D. M. & Rind, D. Does the ocean–atmosphere system have more than one stable mode of operation? *Nature* **315**, 21–26 (1986).
5. Stanley, G. D. Jr & Cairns, S. D. Constructional azooxanthellate coral communities: an overview with implications for the fossil record. *Palaios* **3**, 233–242 (1988).
6. Druffel, E. R. M., King, L. L., Belostock, R. A. & Duessler, K. O. Growth rate of a deep-sea coral using Pb-210 and other isotopes. *Geochim. Cosmochim. Acta* **54**, 1492–1500 (1990).
7. Mikkelsen, N., Erlenkeuser, H., Killingley, J. S. & Berger, W. H. Norwegian corals: radiocarbon and stable isotopes in *Lophelia pertusa*. *Boreas* **11**, 163–171 (1982).
8. Emiliani, C., Hudson, J. H., Shinn, E. A. & George, R. Y. Oxygen and carbon isotopic growth records in a reef coral from Florida Keys and a deep-sea coral from Blake Plateau. *Science* **202**, 627–629 (1978).
9. Smith, J. E. et al. Isotopic analyses of deep-sea corals yield climatic information after compensating for kinetic isotope effects. (Spring Meeting Suppl.) *Eos* **77**, S160 (1996).
10. McConnaughey, T. A. C-13 and O-18 isotopic disequilibrium in biological carbonates 1: Patterns. *Geochim. Cosmochim. Acta* **53**, 151–163 (1989).
11. McConnaughey, T. A., Burdett, J., Whelan, J. F. & Paull, C. K. Respiration and photosynthesis: effects on the carbon-13 content of biological carbonates. *Geochim. Cosmochim. Acta* **61**, 611–622 (1997).
12. Grossman, E. L. & Ku, T. L. Oxygen and carbon isotope fractionation in biogenic aragonite: temperature effects. *Chem. Geol.* **59**, 59–74 (1986).
13. Keen, C. E. *Cruise Report, C.S.S. Hudson 78-020* (Atlantic Geoscience Centre, Dartmouth, 1978).
14. Schumacher, H. & Zibrowius, H. What is hermatypic? A redefinition of ecological groups in corals and other organisms. *Coral Reefs* **4**, 1–9 (1985).
15. Smith, J. E. Late Quaternary climate reconstruction using the deep-water coral *Desmophyllum cristagalli*. *Geol. Surv. Canada Open File* **2950** (1994).
16. Broecker, W. S. et al. Radiocarbon age of waters in the deep Atlantic. *Glob. Biogeochem. Cycles* **4**, 103–117 (1990).
17. Lehman, S. J. & Keigwin, L. D. Sudden changes in North Atlantic circulation during the last deglaciation. *Nature* **356**, 757–762 (1992).
18. Woillard, G. M. & Mook, W. D. C-14 dates at Grande Pile: correlation of land and sea chronologies. *Science* **215**, 159–161 (1982).
19. Coope, G. R. Fossil coleopteran assemblages as sensitive indicators of climate change during Devensian (last) cold stage. *Phil. Trans. R. Soc. B* **280**, 313–340 (1977).
20. Lehman, S. J., Wright, D. G. & Stocker, T. F. *Global Climate Change Vol. 12, Ice in the Climate System* 187–209 (NATO ASI Ser. I, Kluwer Academic Norwell, 1993).
21. Boyle, E. A. Cadmium: chemical tracer of deepwater paleoceanography. *Paleoceanography* **3**, 471–489 (1988).
22. Yu, E.-F., Francois, R. & Bacon, M. P. Similar rates of modern and last-glacial ocean thermohaline circulation inferred from radiochemical data. *Nature* **379**, 689–694 (1996).
23. Samthein, M. et al. Changes in east Atlantic deepwater circulation over the last 30,000 years: eight time slice reconstructions. *Paleoceanography* **9**, 209–267 (1994).
24. Labeyrie, L. D. et al. Changes in the vertical structure of the North Atlantic ocean between glacial and modern times. *Quat. Sci. Rev.* **11**, 301–413 (1992).
25. Oppo, D. W. & Lehman, S. J. Mid-depth circulation of the subpolar North Atlantic during the last glacial maximum. *Science* **259**, 1148–1152.
26. CLIMAP The last interglacial ocean. *Quat. Res.* **21**, 123–224 (1984).
27. Alley, R. B. et al. Abrupt increase in Greenland snow accumulation at the end of the Younger Dryas event. *Nature* **342**, 527–529 (1993).
28. Fairbanks, R. G. A 17,000 years glacio-eustatic sea-level record: influence of glacial melting rates on the Younger Dryas event and deep ocean circulation. *Nature* **342**, 637–642 (1989).
29. Blanchon, P. & Shaw, J. Reef drowning during the last deglaciation: evidence for catastrophic sea-level rise and deep-ocean circulation. *Geology* **23**, 4–8 (1995).
30. Dunbar, R. B. & Cole, J. E. Coral records of ocean–atmosphere variability. *Special Rep. No. 10* (NOAA Climate and Global Change Program, Silver Springs, MD, 1993).

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Hydrothermal gold mineralization in the Witwatersrand basin

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The origin of the gold mineralization in the Witwatersrand basin of South Africa—the largest known gold province—has been controversial for decades, with arguments favouring detrital^{1,2} (placer), modified placer^{3,4} and hydrothermal^{5,6} origins. Here we present the results of an extensive geological study of Witwatersrand rocks which show that the gold (and associated uranium) mineralization is hydrothermal in origin and post-dates a regional high-temperature alteration event. Alteration processes identified on a small scale can be mapped out regionally as roughly strata-bound zones of acid metasomatism extending far into the basin: the fluid flow responsible for this alteration was concentrated in small-scale structures localized along lithological boundaries. We find that the gold precipitated as a consequence of interactions of the fluid with shale-derived hydrocarbons present within the basin.

The sedimentary rocks of the $350 \times 200 \text{ km}$ Witwatersrand basin (Fig. 1) lie unconformably on a $>3.1\text{-Gyr-old}^4$ granite-greenstone basement. Basement granitic magmatism continued during basin filling ($3.1\text{--}2.7 \text{ Gyr ago}$)⁴. The basin fill comprises sedimentary and lesser volcanic material of the Archaean Dominion, West Rand and Central Rand groups and the Ventersdorp Supergroup (Fig. 1). Gold mineralization is concentrated in certain conglomeratic horizons ('reefs') immediately overlying interformational unconformities in the West Rand and more especially the Central Rand groups which are dominated by clastic sedimentary rocks with restricted volcanic intervals. The Central Rand Group is bounded above by Klipriviersberg Group lavas, the oldest members of the Ventersdorp Supergroup. This is overlain by the Proterozoic Transvaal Supergroup. Major gold fields occur along the northern and western margins of the basin where Central Rand rocks are within 4,000 m of the surface.

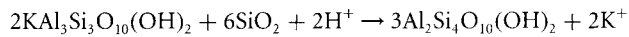
The structural history of the basin is complex⁷. Dominion-age rifting was followed by generally southeast-verging syn-Central Rand thrusting along the northern and western margins of the basin. The Central Rand Group thus occupies a remnant foreland basin. Half-grabens were formed by extension during late Ventersdorp (Platberg) times. Before Transvaal Supergroup sedimentation, folding and thrusting occurred. Finally, post-Transvaal compression occurred associated with uplift of the Vredefort dome⁷.

Structural examination of underground exposures, drill cores and detailed petrographical investigations by scanning electron microscopy (SEM) have revealed the complex post-depositional history of the mineralized rocks. Back-scattered SEM and SEM-based cathodoluminescence on polished sections enables one to examine intact mineral and rock fabrics so that the relative timing of their growth can be determined. Previous SEM-based studies² have

looked only at separated grains, precluding the examination of inter-grain relationships. Detailed examination of Central Rand rocks across the basin have produced a four-stage paragenetic interpretation (Fig. 2) outlined below:

Stage 1. Diagenesis, involving point-contact fracturing, quartz overgrowth, illite (now muscovite), chlorite and pyrite growth.

Stage 2. High-temperature alteration and deformation in which pyrophyllite-rich material developed after muscovite. Phyllosilicate-rich, millimetre to centimetre thick shear zones ('phylionites') formed sub-parallel to bedding, commonly at lithological contacts. Pyrophyllite formed via the reaction



consuming substantial quantities of silica, causing the corrosion of quartz (Fig. 3a). Pyrophyllite generation thus resulted from the interaction of the rock with an acid fluid. The most intense alteration zones, containing pure pyrophyllite, are marked by compressional deformation. These commonly occur at the base or

top of conglomeratic units. Internal fabrics and cross-cutting relationships with respect to the stratigraphy show that these zones form part of a low-displacement thrust system. Pyrophyllite stability indicates that temperatures exceeded 300 °C at pressures of 2.5–3.5 kbar (from fluid inclusion and mineral stability data⁸).

Stage 3. Mineralization is characterized by sub-horizontal fractures filled with uraninite, hydrocarbon, quartz, kaolinite, pyrite, pyrrhotite, gersdorffite, arsenopyrite, galena and gold (Fig. 3b–d) in conglomeratic units in close proximity to phylionites. The full intensity of fracturing is only apparent using cathodoluminescence. Uraninite grew after phyllosilicates developed during the high-temperature alteration stage and also filled fractures associated with hydrocarbon (Fig. 3d, e). These observations demonstrate the epigenetic nature of uraninite. Gold occurs within a few centimetres of hydrocarbon seams and none of over 40,000 gold grains examined in this extensive study occurs in textural sites present at the time of sedimentation. Gold grains studied *in situ* were precipitated late in the paragenesis, in association with

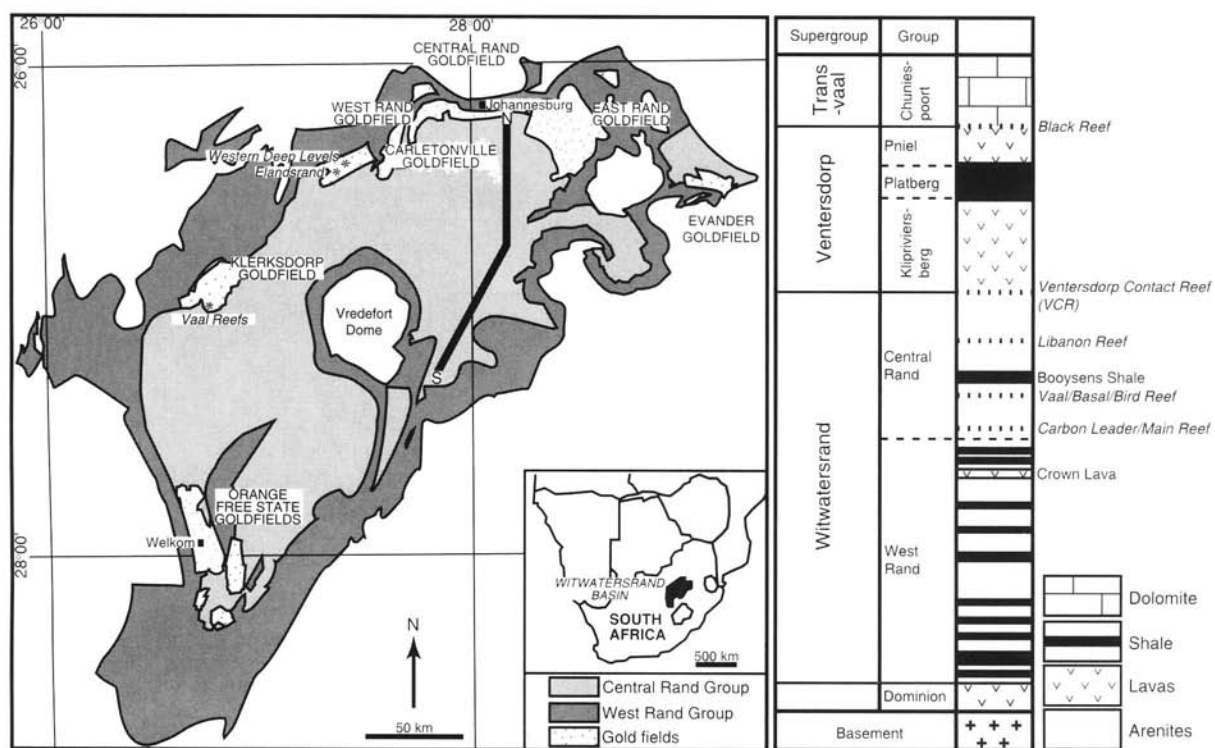


Figure 1 The geology of the Witwatersrand basin, showing the major goldfields and mines referred to in the text and a simplified stratigraphic column. The line of the section shown in Fig. 4 is also marked.

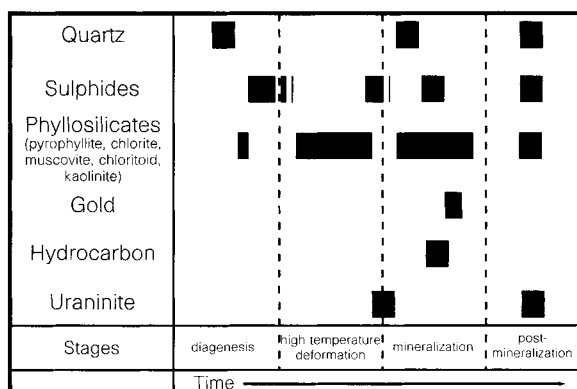


Figure 2 Simplified post-depositional history of Witwatersrand reef material based on observations carried out on 141 thin sections (from 86 samples) from the Vaal reef in the Klerksdorp goldfield, the Basal reef near Welkom and the Carbon leader and the Ventersdorp contact reef in the Carletonville goldfield.

hydrocarbon and during fracturing (Fig. 3). Temperatures were lower than in stage 2.

Stage 4. Post-mineralization events include localised fracturing, veining, further shearing and pressure solution which vary in intensity across the basin.

All of the textures described here are interpreted as resulting from hydrothermal processes, a conclusion reached by previous workers^{9,10}. However, many consider the gold in Witwatersrand rocks to be predominantly detrital in origin¹⁻⁴. Evidence cited in support of a detrital origin for gold mainly comes from separated-grain studies and includes the round form of many grains, and grain

surface morphologies considered to be the result of abrasion². It is clear that an understanding of the context of mineral grains within the rock is essential for the correct interpretation of its origin (Fig. 3)¹¹.

Observations of hydrocarbon present within the mineralized rocks are critical in determining the nature and timing of gold mineralization. Witwatersrand 'carbon' is residual hydrocarbon in the form of pyrobitumen. The pyrobitumen has a proto-graphitic form called 'mesophase'¹², which is characterized by a strongly anisotropic structure, and occurs in these rocks mainly as elongate bodies, termed 'spindles' (Fig. 3f). It is clear that the hydrocarbon

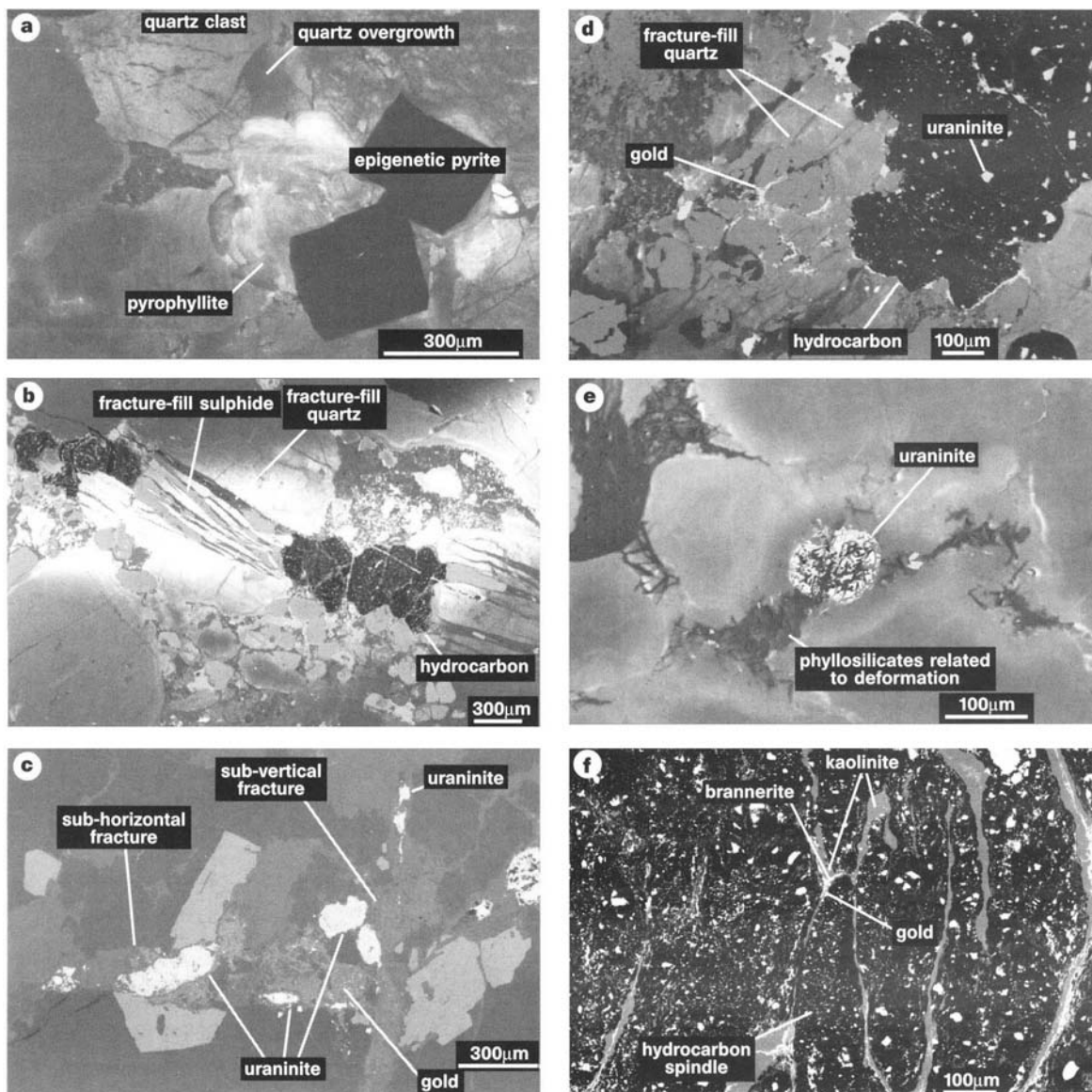


Figure 3 SEM photomicrographs. **a**, Pyrophyllite infilling porosity created by the corrosion of quartz grains and overgrowths. This image also shows epigenetic pyrite post-dating detrital quartz clasts. **b**, Sub-horizontal fractures within quartz grains, filled by secondary quartz, sulphides and kaolinite. The fractures formed coevally with the introduction of hydrocarbon, as the fractures form tip zones to the terminations of hydrocarbon seams and the hydrocarbon invades fracture openings in quartz clasts. **c**, Sub-horizontal fracture containing uraninite, allanite, chlorite and gold (white specks). Uraninite also occurs in paragenetically late sub-vertical fractures. **d**, Sub-horizontal quartz/gold-filled fractures associated with hydrocarbon. **e**, Uraninite overgrowing phyllosilicates of the high-temperature deformation stage. Thus, despite its rounded morphology, the uraninite is epigenetic. **f**, Hydrocarbon spindles in a hydrocarbon seam. The spindles are

elongated parallel to the margins of the sub-horizontal seam. The interspindle pore-space is filled by kaolinite, brannerite and gold. Gold occurs between and around the hydrocarbon spindles associated with gersdorffite and precipitated subsequent to uraninite and hydrocarbon introduction, during mesophase development¹³, but as part of the same complex mineralizing event. The gold grains have a complex morphology, with thin appendages along grain and hydrocarbon spindle boundaries. Folded appendages on gold grains have been interpreted as a result of 'peening'. Only minor mechanical damage to these grains during separation would be necessary to produce apparently 'peened' form. Kaolinite stability and fluid-inclusion data indicate lower-temperature conditions during the mineralization stage than during the high-temperature stage but similar pressures.

was once liquid^{4,9,12} as it invaded pre-existing fractures (Fig. 3d). Mesophase pyrobitumen formation resulted from initial radiation-induced free-radical polymerization by uraninite which stabilized migrating basinally derived liquid hydrocarbon in the fracture network. Oil probably resided in voids in the surrounding rocks before fracturing where some remains as 'flyspeck'. The recognition of mesophase pyrobitumen and the localization of hydrocarbon in post-primary porosity and fractures renders untenable an origin as *in situ* algal material^{2,13}. It is the mesophase texture that has been erroneously described in the literature as 'organic' structure.

Previous work has shown that all Witwatersrand goldfields are characterized by similar peak pressure-temperature conditions. Pyrophyllite occurs widely^{8,10} in the reefs and their immediate country rocks. The basin-wide distribution of alteration minerals shows a clearly defined pattern (Fig. 4) with zones of intense alteration concentrated along the basin margins, extending 20–100 km into the basin around specific unconformities. The alteration, although generally strata-bound, does cross-cut the stratigraphy in places (Fig. 4). These new data demonstrate clearly that the Witwatersrand basin has experienced regional-scale hydrothermal fluid flow^{6,8,10}. Phyllonites in the most-altered rocks, which comprise pure pyrophyllite, rule out an origin by metamorphism of detrital kaolinite. These two observations preclude a palaeosol origin for these aluminous zones¹⁴.

In our model for hydrothermal mineralization, we envisage that the diagenesis of moderately mature sediments led to the replacement of detrital feldspar and iron-rich phases by muscovite and pyrite. During compressive deformation hydrothermal fluids,

which caused hydrogen metasomatism, were focused along small-scale shear zones and fracture swarms whose positions were controlled by the lithological architecture. The distal parts of larger thrust systems, characterized by low displacements, developed along small mechanical heterogeneities such as bedding planes and sedimentary foresets. Permeability development, fluid flow and mineralization are thus to be expected in such sites, and minerals occurring there are not all sedimentary in origin as suggested by some workers^{1,2,15}. Subsequent lower-temperature deformation was restricted to fractures which concentrated fluid flow. Uraninite precipitated from the hydrothermal fluid as a result of reduction by either pre-existing minerals or hydrocarbon. Continued hydrothermal fluid flow and interaction with uraninite caused the alteration of hydrocarbons to pyrobitumen and ultimately mesophase. Reduction of the hydrothermal fluid by the hydrocarbon also caused precipitation of gold.

Objections to hydrothermal models for the origin of mineralization in the Witwatersrand basin have centred on the strong correlation of gold with sedimentary features, and the lack of post-burial pathways for fluids¹⁵. The widespread low-displacement thrust systems marked by phyllonites, and the mineralized fracture systems containing uraninite, hydrocarbon and gold, provide the first evidence of significant structurally controlled fluid migration pathways in the basin. A hydrothermal model also explains the size and geometry of the ore bodies, the range of reef lithologies, their relatively uniform metamorphic grade and the mineralization-pyrobitumen association. Evidence for the post-depositional crystallization of gold, pyrite, uraninite and hydrocarbon (Fig. 3), and

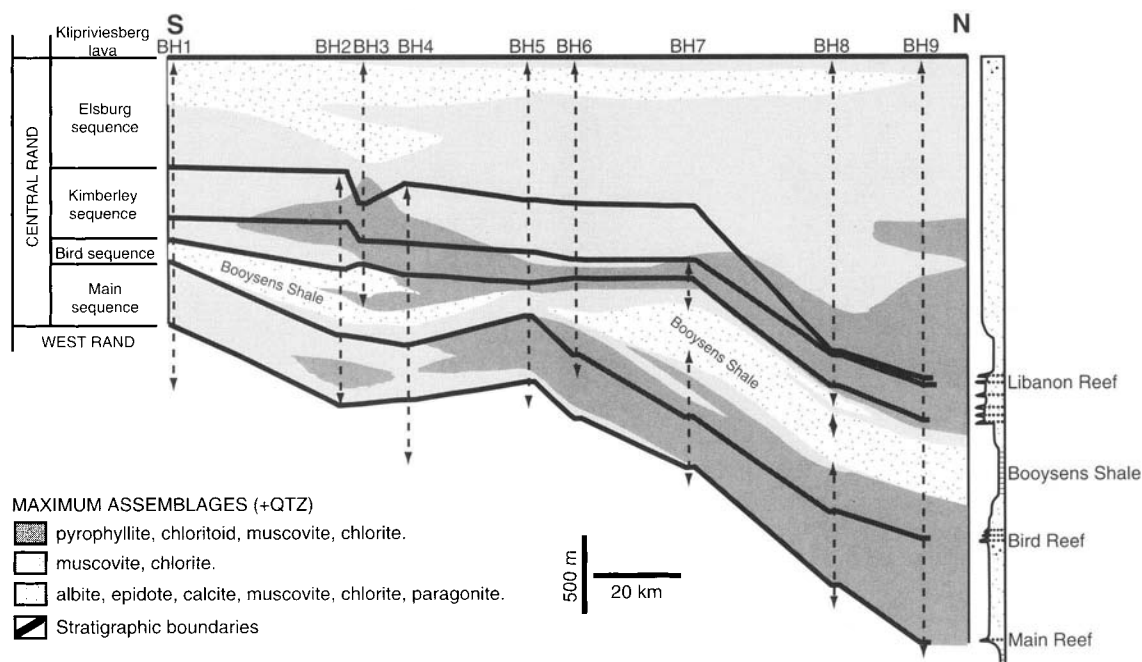


Figure 4 North-south section through the stratigraphy of the Witwatersrand basin along the bold line shown in Fig. 1. The lateral and vertical distribution of alteration zones is shown, based on a combination of over 4,000 X-ray diffraction measurements and 2,000 PIMA (portable infrared mineral analysis) analyses of samples taken every 10–20 m through the boreholes indicated (BH1–9). This study has been undertaken using investigations of >80 drill cores from geographical sites throughout the Witwatersrand basin. The most intense zones of alteration are characterized by the presence of pyrophyllite and chloritoid. Zones

of less-intense alteration, characterized by muscovite and chlorite, then albite and calcite, occur symmetrically both vertically and laterally away from the pyrophyllitic alteration. The alteration records the progressive neutralization of an acid fluid, and intense alteration occurs symmetrically above and below mineralized horizons that follow unconformities. Intense (pyrophyllitic) alteration occurs within both mineralized conglomerate packages and surrounding quartzites for distances of over 500 m above and below the reefs.

hence their origin from solution, is compelling. Uraninite occurring in fractures means that oxidizing solutions must have been present at a late stage in the Witwatersrand rocks. The scale of the regionally extensive alteration zones and their cross-cutting nature with respect to stratigraphy (Fig. 4) indicate that local remobilization^{3,4} of gold and uranium is not responsible for the mineralization described here. There is no evidence for the rapid, small-scale variations in fluid and mineral chemistry that are a thermodynamic necessity to permit such a redistribution. □

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1. Minter, W. E. L. *Can. Soc. Petrol. Geol. Mem.* 5, 801–829 (1978).
2. Hallbauer, D. K. in *Mineral Deposits of Southern Africa* (eds Anhaeusser, C. R. & Maske, S.) 731–752 (Geol. Soc. S. Africa, Johannesburg, 1986).
3. Frimmel, H. E., Le Roux, A. P., Knight, J. & Minter, W. E. L. *Econ. Geol.* 88, 249–265 (1993).
4. Robb, L. J. & Meyer, F. M. *Ore Geol. Rev.* 10, 67–94 (1995).
5. Phillips, G. N., Myers, R. E. & Palmer, J. A. *Geology* 15, 1027–1030 (1987).
6. Phillips, G. N. & Myers, R. E. *Econ. Geol. Monogr.* 6, 597–607 (1989).
7. Coward, M. P., Spencer, R. M. & Spencer, C. E. *Early Precambrian Processes*, 243–269 (Spec. Publ. 95, Geol. Soc. Lond., 1995).
8. Phillips, G. N. & Law, J. D. M. *Ore Geol. Rev.* 9, 1–31 (1994).
9. Schidolowski, M. *US Geol. Surv. Prof. Pap.* 1161N, 1–29 (1981).
10. Phillips, G. N. *J. Metam.* 6, 311–332 (1988).
11. Minter, W. E. L., Geodhart, M., Knight, J. & Frimmel, H. E. *Econ. Geol.* 88, 237–248 (1993).
12. Gray, G. J., Lawrence, S. R., Kenyon, A. K. & Cornford, C. J. *Geol. Soc. Lond.* (in the press).
13. Ebert, L. B. *et al.* *Ore Geol. Rev.* 5, 423–444 (1990).
14. Holland, H. D. & Feakes, C. R. *J. Geol.* 97, 761–762 (1989).
15. Smith, N. D. *Geology* 17, 91–92 (1989).

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Subgenual prefrontal cortex abnormalities in mood disorders

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Pathological disturbances of mood may follow a 'bipolar' course, in which normal moods alternate with both depression and mania, or a 'unipolar' course, in which only depression occurs^{1–3}. Both bipolar and unipolar disorders can be heritable illnesses associated with neurochemical, neuroendocrine and autonomic abnormalities. The neurobiological basis for these abnormalities has not been established^{2,3}. Using positron emission tomographic (PET) images of cerebral blood flow and rate of glucose metabolism to measure brain activity, we have now localized an area of abnormally decreased activity in the prefrontal cortex ventral to the genu of the corpus callosum in both familial bipolar depressives and familial unipolar depressives. This decrement in activity was at least partly explained by a corresponding reduction in cortical volume⁴, as magnetic resonance imaging (MRI) demonstrated reductions in the mean grey matter volume in the same area of 39 and 48% in the bipolar and

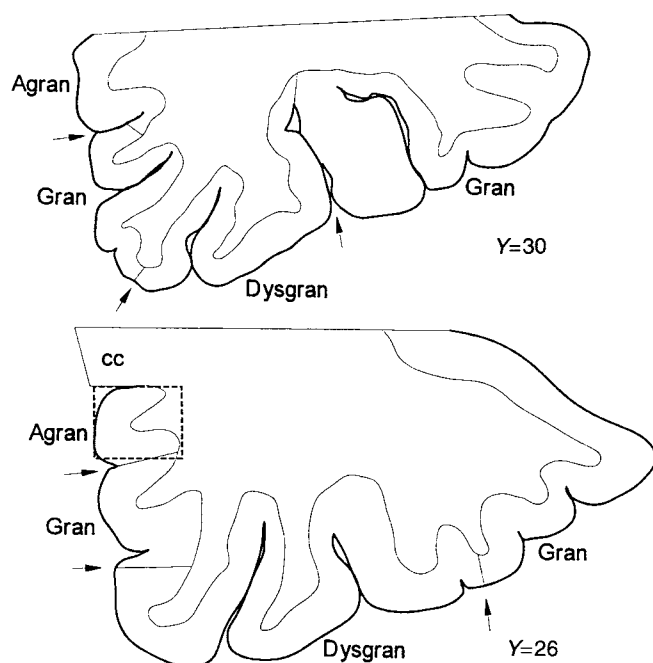


Figure 1 Drawings of coronal sections through the genu of the corpus callosum (CC) and subgenual and posterior orbital PFC of a human brain at levels 26 and 30mm rostral to the anterior commissure. Agranular ('agran'), dysgranular ('dysgran') and granular ('gran') cortical regions are marked. The subgenual PFC consists of agranular cortex on the anterior cingulate gyrus immediately ventral to the genu of the corpus callosum³⁰. Dashed line indicates the centre of the smaller ROI used for regional measures in the 953B images. This cylindrical ROI, 1 cm in diameter and spanning 1–9 mm ventral to the bicommissural plane, was placed²⁹ *a priori* in each subject's image over the coordinates of the peak $|t|$ value identified in the subgenual PFC in the t image from group A: $x = 5$, $y = 25$, $z = -6$ (in mm from the anterior commissure, with positive x as right, positive y as rostral, and negative z as ventral to the bicommissural plane²⁸).

unipolar samples, respectively. This region has previously been implicated in the mediation of emotional and autonomic responses to socially significant or provocative stimuli, and in the modulation of the neurotransmitter systems targeted by antidepressant drugs^{3,5–10}.

Low-resolution PET images of cerebral blood flow (BF) (full width at half-maximum (FWHM), 17 mm) from unmedicated, bipolar depressives and from controls were divided into two groups (A and B) for hypothesis generation and subsequent testing (Table 1). Using the images from group A, a statistical image composed of unpaired t values (the difference between the mean values for each subject sample divided by the standard error of this difference) was computed by subtracting the mean blood flow for the controls from that of the depressives at every voxel (volume element) and then dividing the resulting differences by the local variance¹¹. Regions-of-interest (ROI) were defined in this t -image, which highlighted areas where blood flow inherently differed between the bipolar-depressives and controls from group A (ref. 11). The ROI containing the largest absolute difference in mean flow between groups was defined in the subgenual prefrontal cortex, where flow was decreased by 7.7% in the depressives relative to the controls from group A (the proximity of this abnormality to the midline precluded laterality determinations from the PET image data; Fig. 2 legend). The hypothesis that blood flow was abnormal in bipolar depression in this ROI was confirmed by comparing regional measures from the independent subject group B, in which mean flow was decreased by 6.6% in the depressives relative to the controls ($t = -4.3$, corrected d.f. = 16, corrected $P(1\text{-tailed}) < 0.01$).

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Table 1 PET image comparison of regional BF and metabolic rates for bipolar depressed, unipolar depressed, bipolar manic and control patients

Hypothesis:	Generating		Testing		Testing and extending		Extending	Extending
Subject group:	A		B		C		FPDD	Manic
PET camera	PETT VI		PETT VI		Siemens CTI 953B		953B	953B
Measure	Relative BF		Relative BF		Relative BF and MRglu		Relative MRglu	Relative MRglu
Subsample:	BP-D	Control	BP-D	Control	BP-D	Control		
<i>n</i>	6	24	5	15	7	12	10	4
Mean age, yr ($\pm$ s.d.)	29 $\pm$ 5.5	29 $\pm$ 7.1	27 $\pm$ 6.5	27 $\pm$ 8.5	37 $\pm$ 8.7	33 $\pm$ 11	39 $\pm$ 7.3	30 $\pm$ 11
Right-handed (%)	100	100	100	100	86	92	100	100
Conclusion, subgenual PFC activity:			BF is reduced in BP-D versus control		BF and MRglu are both reduced in BP-D versus control		MRglu is reduced in FPDD versus control	MRglu appears raised in manic versus control, BP-D and FPDD

For abbreviations, see Methods.

To localize this abnormality more precisely, glucose metabolism images were acquired in a third, independent set of unmedicated, bipolar depressives and controls (group C in Table 1) using a higher-resolution camera (Siemens/CTI 953B; reconstructed resolution, 5 mm FWHM). A smaller ROI was stereotactically positioned in each group C image at the coordinates of the $|t|$ value identified in the subgenual prefrontal cortex in the t -image from group A (Fig. 1). Mean normalized metabolism was decreased 16.3% and mean blood flow was decreased 18.5% in this ROI in the bipolar depressives relative to the controls (mean $\pm$ one standard deviation for metabolism = 0.87 ± 0.15 and 1.04 ± 0.15 , respectively; $t = -2.3$, corrected d.f. = 12, $P(1\text{-tail}) < 0.025$; and for blood flow = 1.01 ± 0.15 and 1.24 ± 0.17 , respectively, $t = -3.0$, corrected d.f. = 13, $P(1\text{-tail}) < 0.01$). The greater magnitude and variance of the differences measured in this sample was probably accounted for by the higher spatial resolution of the images.

The same high-resolution PET methods were applied to unipolar depressives with familial pure depressive disease (FPDD; criteria that exclude depressives with a family history of mania¹²) and to bipolar subjects scanned in the manic phase¹. The unipolar group ($n = 10$; 7 female) met criteria for recurrent, major depressive disorder¹ and FPDD¹², and their mean age, depression severity (mean Hamilton Depression Rating Scale (HDRS) score, 25.5 ± 5.4) and medication free period (>4 weeks) were similar to those of bipolar group C (Table 1). The mean subgenual prefrontal cortex metabolism in the unipolar depressives (0.91 ± 0.11) was decreased 12.2% relative to the controls ($t = -2.3$, d.f. = 20, $P(1\text{-tail}) < 0.025$) and did not significantly differ from that of the bipolar depressives. Previously, the only functional neuroimaging finding consistently identified in both bipolar and unipolar depressives was located in the dorsolateral prefrontal cortex where reduced activity is thought to correlate with the neuropsychological manifestations of depression (for example, slowing of thought speed)^{13–20}.

The manic subjects ($n = 4$) met criteria for bipolar disorder–manic phase¹ (mean Mania Rating Scale²¹ score was 20 ± 4.4 , one female, one medicated within 4 weeks). In contrast to the findings in the depressed groups, the mean metabolism in the manic group (1.15 ± 0.054) was increased relative to that of the control ($t = 2.2$, d.f. = 12, $P(2\text{-tail}) < 0.05$), bipolar-depressed ($t = 4.6$, $P < 0.01$) and unipolar-depressed groups ($t = 5.5$, $P < 0.01$). Consistent with these differences, in one subject scanned in both phases, normalized metabolism in the subgenual prefrontal cortex was 0.84 while depressed and 1.11 eight weeks later while manic. Although these preliminary observations in mania require replication in a larger sample, they suggest that subgenual prefrontal cortex metabolism is mood-state dependent.

Decreased blood flow and metabolism in the subgenual prefrontal cortex in depression could reflect a regional reduction in either synaptic activity²² or tissue volume (because of the low spatial resolution of PET, reductions in tissue associated with atrophy or hypoplasia reduce the magnitude of local blood flow and metabolic measures from PET images, a phenomenon known as the partial

volume averaging effect)⁴. The grey matter volume of the corresponding cortex was therefore measured in MRI images acquired in mood-disordered and control subjects (Figs 2, 3). MRI scans were not available for the subjects in groups A and B, so to improve the sensitivity for detecting intergroup differences (regional brain volume measures have greater variability than PET measures), the sample sizes were increased by adding MRI data from subjects not involved in the PET comparisons. The expanded bipolar group included the unmedicated, bipolar depressives from group C together with two medicated-depressed, four manic and eight remitted (six medicated) subjects with bipolar disorder¹ (total $n = 21$; age = 35 ± 8.2 ; 13 female; 20 right-handed; all had a parent or sibling with bipolar disorder). The expanded unipolar group combined the FPDD group with seven additional unipolar depressives who met criteria for recurrent, major depressive disorder¹, but not for FPDD¹² (total $n = 17$; mean age = 35 ± 9.4 ; 10 female, 16 right-handed). Additional controls were combined with those from group C (total $n = 21$; age = 34 ± 8.2 ; 11 female; 19 right-handed).

The left subgenual prefrontal cortex grey matter volume was reduced by 39% in the bipolar subjects and 48% in the unipolar depressives relative to the controls ($F = 9.8$; $P < 0.0002$, after covarying for age, gender and whole brain volume; Fig. 3), and did not significantly differ between the unipolar and bipolar groups. (Notably, post hoc tests showed that the volumetric differences identified in the expanded group of bipolar subjects were also significant when the controls were compared either to the seven bipolar subjects from group C ($P < 0.05$) or to the 14 bipolar subjects not included in group C ($P < 0.005$).) No significant differences in mean volume were found between the bipolar and control groups in other portions of the anterior cingulate gyrus, including the right subgenual prefrontal cortex (219 ± 95 and $209 \pm 66 \text{ mm}^3$, respectively) or the dorsal anterior cingulate cortex (left: 418 ± 184 and $389 \pm 211 \text{ mm}^3$, respectively, $t = 0.47$; right: 456 ± 134 and $406 \pm 134 \text{ mm}^3$, respectively, $t = 1.2$). Post hoc analysis indicated that the volumetric difference in the subgenual prefrontal cortex is present irrespective of mood state in bipolar disorder (mean values for the symptomatic (manic or depressed) and remitted subsamples were 131 ± 72 and $146 \pm 99 \text{ mm}^3$, respectively). The anatomical abnormality also persisted during antidepressant drug treatment in 15 of the unipolar depressives who were reimaged following a mean treatment period of 3.2 ± 3.5 months (the mean volume was $126 \pm 66 \text{ mm}^3$ pre-treatment and $123 \pm 61 \text{ mm}^3$ post-treatment). Thus, the reduction in the left subgenual prefrontal cortex grey matter volume in mood disorders, localized by synergistic use of PET and MRI image data, could reflect either an abnormality of brain development related to the tendency to develop mood episodes or a degenerative change resulting from recurrent illness.

In monkeys and other experimental animals, the subgenual prefrontal cortex has extensive connections with structures implicated in emotional behaviour and autonomic/neuroendocrine responses to stressors, including the amygdala, the lateral hypothal-

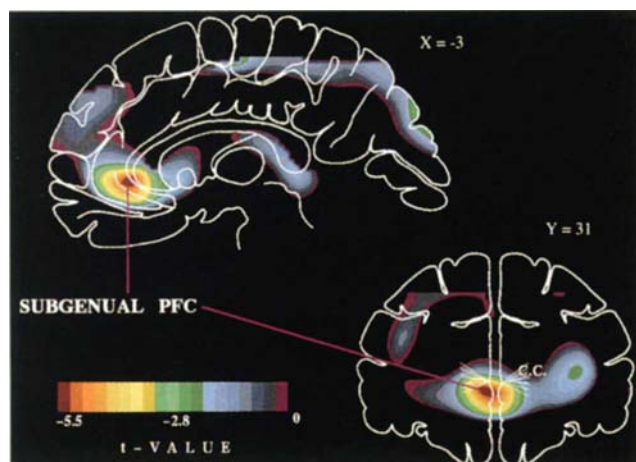


Figure 2 Coronal ($y = 31$ mm) and sagittal ($x = -3$ mm) sections showing negative voxel t values where metabolism is decreased in depressives relative to controls. This image, generated *post hoc* to provide optimal localization of the subgenual PFC abnormality, compares all unmedicated depressives scanned with the 953B (bipolar depressives from group C plus unipolar depressives with FPDD) with the group C controls. The stereotaxic centre-of-mass of this difference ($x = -2, y = 32, z = -2$) lay within one FWHM (17 mm for PET VI) from the peak BF difference in group A (Fig. 1) and from the centre-of-mass of the BF difference computed *post hoc* for the entire PET VI image set (groups A plus B): $x = 1, y = 25, z = -6$. All three coordinate sets localize to the agranular region of the anterior cingulate gyrus ventral to the CC (Fig. 1). The spatial resolution of PET precludes clear laterality distinctions. Anterior, or left, is to the left.

lamus, the nucleus accumbens, and the brainstem serotonergic, noradrenergic and dopaminergic nuclei (the function of these neurotransmitter systems appears to be blunted during depressive episodes and to be augmented by antidepressant drug treatments)^{2,3,5-7}. Humans with lesions that include the subgenual prefrontal cortex demonstrate abnormal autonomic responses to emotional experiences, inability to experience emotion related to concepts that ordinarily evoke emotion, and impaired comprehension of the adverse consequences of pernicious social behaviours^{8,9}. Based partly upon these observations, Damasio *et al.*^{8,10} proposed that the ability to discern the outcome of social behaviour in terms of punishment and reward depends upon visceral feedback mediated through interactions between the ventromedial prefrontal cortex and autonomic centres. If so, disordered interactions between the subgenual prefrontal cortex and interconnected structures may be related to the pathological guilt or anxiety characterizing depression, or the rapid shifts between inappropriate euphoria and anger seen in mania^{2,3}. The neural network formed by these structures may contain a disease process that is not confined to the subgenual prefrontal cortex, as reduced left amygdala volume²³ and third ventricle enlargement¹⁸ (the subgenual prefrontal cortex shares substantial, predominantly ipsilateral connections with the amygdala and the medial thalamic nuclei surrounding the third ventricle⁵) have also been reported in bipolar disorder. Post-mortem histopathological assessments within this circuitry may elucidate the neurobiology of familial mood disorders. □

Methods

Subjects. Characteristics of subgroups appear in Table 1. The bipolar-depressed (BP-D) subjects met criteria for bipolar disorder, depressed phase¹, and had a parent or sibling with probable or definite bipolar disorder. Hamilton Depression Rating Scale (HDRS) scores (21 items)²⁴ were in the moderately-to-severely-depressed range (mean = 24.2 ± 3.5 (20–29) for groups A and B, and 23.4 ± 6.5 (16–35) for group C). Subjects had not received psychotropic or

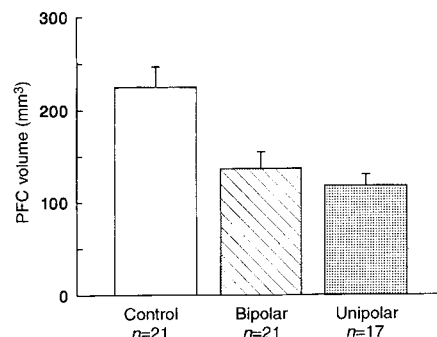


Figure 3 MRI-based volumes of the left subgenual prefrontal cortex (PFC) grey matter differed between the bipolar disordered, unipolar depressed and control groups (ANOVA, $F = 9.8$; $P < 0.0002$, co-varying for gender, age and whole brain volume). Post hoc tests (Tukey-Kramer) showed significant volumetric decreases in the bipolar and unipolar groups relative to the control group. Whole-brain MRI volume and skull X-ray measures (product of anterior-posterior distance between the inner tables of the skull at the estimated bicommissural segment²⁹ and perpendicular distance from the midpoint of the bicommissural segment to the vertex²⁹) were slightly smaller in the unipolar group compared with the bipolar and control groups, and did not differ between the bipolar and control groups. The left subgenual PFC/whole brain volume ratio was also reduced in the bipolar and unipolar groups, being $1.8 \times 10^{-4} \pm 0.74 \times 10^{-4}$, $1.1 \times 10^{-4} \pm 0.64 \times 10^{-4}$, and $1.1 \times 10^{-4} \pm 0.51 \times 10^{-4}$ in the control, bipolar, and unipolar groups, respectively ($F = 8.4$; $P < 0.001$).

other drugs likely to alter blood flow (BF) or glucose metabolism for >4 weeks. Exclusion criteria included substance abuse within one year and major medical disorders.

Controls met the same exclusions and denied having had major psychiatric disorders. Group A controls consisted of 24 subjects involved in our previous study of unipolar depression¹¹, who also denied a family history of psychiatric disorders. This large, carefully screened control group was used to generate the composite normative image for the t -image computation. Group B controls were matched with bipolar subjects in group B (40% female in each) and group C controls with the bipolar subjects in group C (50 and 57% female, respectively) for age, handedness and gender.

Image acquisition. PET scans were acquired as subjects rested with eyes closed. For groups A and B, images were obtained using PET VI and 60–80 mCi of $H_2^{15}O$ (ref. 25). Tissue radioactivity was measured, which over the range tested accurately reflects differences in regional BF²⁶.

For group C, images were acquired with a Siemens 953B tomograph (resolution was 5 mm FWHM, septa inserted). Glucose metabolism was measured using 5–10 mCi of [^{18}F]2-fluorodeoxyglucose, adaptations of the Sokoloff method²⁷ and arterial blood sampling. A linear normalization was applied by dividing regional-by-global metabolic rate for glucose (MRglu; mean global values did not significantly differ across the control, bipolar-depressed, and unipolar-depressed (FPDD) groups, being 6.0 ± 0.95 , 6.2 ± 1.2 and 5.7 ± 1.1 mg per 100 mg per min, respectively). The metabolic images were not filtered (that is, FWHM was 5 mm). Regional BF was also measured²⁵ and the reconstructed BF images were filtered to a 3-dimensional resolution of 14 mm FWHM. Technical problems precluded acquisition of the metabolic image for one bipolar depressive and the BF image for another.

Image analysis. To guide regional analyses, PET scans from group A were used to compute a composite statistical image highlighting differences between bipolar depressives and controls. After remapping primary images into a common, stereotaxic array^{28,29}, an image of unpaired t -values was computed, with each voxel representing the difference in mean BF between depressives and controls divided by the local variance¹¹. The t image was searched for t values above a threshold $|t|$ that would correspond to $P < 0.05$ in a single test. ROI

were defined to encompass the voxel containing the peak $|t|$ value and adjacent voxels with similar t values. The size of each ROI was optimized by computing regional t for progressively larger ROI, and the ROI for which the regional t was maximized was selected. Eleven ROIs were defined, located in the following regions (per cent difference in mean normalized flow in depressives relative to controls appears in parentheses): subgenual prefrontal cortex (PFC) (−7.7%), right (R) insula (−7.5%), R and left (L) temporoparietal cortex (−7.4 and −6.7%, respectively), R and L lentiform nucleus (−6.3 and −5.7%, respectively), rostral thalamus (−6.1%), posterior cingulate (−5.6%), L hippocampus (−5.2%), L frontal pole (4.4%) and R precuneate (4.5%).

The hypothesis that BF differed between depressives and controls in each ROI was tested by comparing the mean BF of the depressed and control samples from group B in one-tailed t -tests, corrected for multiple comparisons (Bonferroni), with degrees of freedom corrected for unequal variance (Satterthwaite). Thus, the ROI used to obtain the BF measures for both groups A and B were identical in size and location, having been generated from the t -image from group A, and stereotactically placed in each subject's PET image^{11,28,29}. The only mean BF difference between the depressives and controls from group B achieving significance after corrections from 11 tests was found in the subgenual PFC.

Because of the higher spatial resolution of the 953B images, regional measures in group C were obtained using the smaller ROI shown in Fig. 1. Thus, they were not guided by information regarding inherent differences between the depressives and controls from groups B or C.

Neuromorphometric measures. MR images acquired using a Siemens VISION 1.5T scanner and a 3D MPRAGE sequence (T1, 300 ms; TR, 9.7; TE, 4; flip angle, 12°; matrix, 256 × 256 × 128, 1 × 1 × 25 mm voxels) were resliced to 1 mm³ voxels with coronal sections oriented perpendicular to the bicommissural line using ANALYZE. The grey matter was manually traced for the first full gyrus beneath the corpus callosum in all coronal slices between the anteriormost point of the callosum and the anteriormost plane where the internal capsule no longer divided the striatum. Segmentation was performed by one investigator (W.C.D.) blinded to diagnosis. Virtually identical mean intergroup differences were obtained by a second rater (J.R.S.) blinded to the measures of the first. Variance components estimates (intraclass correlation coefficients) showed that 1.0% of the total variability was due to interrater differences and 99% to inter-subject differences.

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1. *Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R)* (American Psychiatric Association, Washington, DC, 1987).
2. Goodwin, F. K. & Jamison, K. R. *Manic-depressive Illness* (Oxford University Press, New York, 1990).
3. Drevets, W. C. & Todd, R. D. in *Adult Psychiatry* (ed. Guze, S. B.) 99–142 (Mosby, St Louis, 1997).
4. Mazziotta, J. C., Phelps, M. E., Plummer, D. & Kuhl, D. E. Quantitation in positron emission computed tomography: 5. Physical-anatomical effects. *J. Comput. Assist. Tomogr.* 5, 734–743 (1981).
5. Carmichael, S. T. & Price, J. L. Limbic connections of the orbital and medial prefrontal cortex in macaque monkeys. *J. Comp. Neurol.* 363, 615–641 (1995).
6. Neafsey, E. J., Terberry, R. R., Hurley, K. M., Ruit, K. G. & Fryszak, R. J. in *Neurobiology of Cingulate Cortex and Limbic Thalamus* (eds Vogt, B. A. & Gabriel, M.) 206–223 (Birkhauser, Boston, 1993).
7. Sesack, S. R., Deutch, A. Y., Roth, R. H. & Bunney, B. S. Topographic organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study using *Phaseolus vulgaris* leucoagglutinin. *J. Comp. Neurol.* 290, 213–242 (1989).
8. Damasio, A. R., Tranel, D. & Damasio, H. Individuals with sociopathic behavior caused by frontal damage fail to respond automatically to social stimuli. *Behav. Brain Res.* 41, 81–94 (1990).
9. Bechara, A., Tranel, D., Damasio, H. & Damasio, A. R. Failure to respond automatically to anticipated future outcomes following damage to the prefrontal cortex. *Cerebr. Cort.* 6, 215–225 (1996).
10. Damasio, A. R. *Descartes's Error: Emotion, Reason, and the Human Brain*. (Grosset/Putnam, New York, 1994; Picador MacMillan, London, 1995).
11. Drevets, W. C. *et al.* A functional anatomical study of unipolar depression. *J. Neurosci.* 12, 3628–3641 (1992).
12. Winokur, G. The development and validity of familial subtypes in primary unipolar depression. *Pharmacopsychiatry* 15, 142–146 (1982).
13. Baxter, L. R. *et al.* Cerebral metabolic rates for glucose in mood disorders. *Arch. Gen. Psychiat.* 42, 441–447 (1985).
14. Baxter, L. R. *et al.* Reduction of prefrontal cortex glucose metabolism common to three types of depression. *Arch. Gen. Psychiat.* 46, 243–250 (1989).
15. Buchsbaum, M. S. *et al.* Frontal cortex and basal ganglia metabolic rates assessed by positron emission tomography with [¹⁸F]2-deoxyglucose in affective illness. *J. Affect. Dis.* 10, 137–152 (1986).
16. Cohen, R. M. *et al.* Evidence for common alterations in cerebral glucose metabolism in major affective disorders and schizophrenia. *Neuropsychopharmacology* 2, 241–254 (1989).
17. Dolan, R. J. *et al.* Dorsolateral prefrontal cortex dysfunction in the major psychoses: symptom or disease specificity? *J. Neurol. Neurosurg. Psychiat.* 56, 1290–1294 (1993).
18. Drevets, W. C. & Botteron, K. in *Adult Psychiatry* (ed. Guze, S. B.) 53–82 (Mosby, St Louis, 1996).
19. Drevets, W. C. & Raichle, M. E. Reciprocal suppression of regional cerebral blood flow during emotional versus higher cognitive processes: implications for interactions between emotion and cognition. *Cognit. Emot.* (in the press).
20. Drevets, W. C., Spitznagel, E. L., MacLeod, A. K. & Raichle, M. E. Discriminatory capability of PET measurements of regional blood flow in familial pure depressive disease. *Abstr. Soc. Neurosci.* 18, 1596 (1992).

21. Young, R. C., Biggs, J. T., Ziegler, V. E. & Meyer, D. A. A rating scale for mania: reliability, validity and sensitivity. *Brit. J. Psychiat.* 133, 429–435 (1978).
22. DiRocco, R. J., Kageyama, G. H. & Wong-Riley, M. T. The relationship between CNS metabolism and cytoarchitecture: a review of [¹⁴C]-deoxyglucose studies with correlation to cytochrome oxidase histochemistry. *Comp. Med. Imag. Graph.* 13, 81–92 (1989).
23. Pearlson, G. D. *et al.* Medial and superior temporal gyral volumes and cerebral asymmetry in schizophrenia versus bipolar disorder. *Biol. Psychiat.* 41, 1–14 (1997).
24. Hamilton, M. A rating scale for depression. *J. Neurol. Neurosurg. Psychiat.* 23, 56–62 (1960).
25. Raichle, M. E., Martin, W. R. W., Herscovitch, P., Mintun, M. A. & Markham, J. Brain blood flow measured with intravenous [¹⁵O]. II. Implementation and validation. *J. Nucl. Med.* 24, 790–798 (1983).
26. Fox, P. T. & Mintun, M. A. Noninvasive functional brain mapping by change distribution analysis of averaged PET images of H₂¹⁵O tissue activity. *J. Nucl. Med.* 30, 141–149 (1989).
27. Phelps, M. E. *et al.* Tomographic measurements of local cerebral glucose metabolic rate in humans with [¹⁸F]-2-fluoro-2-deoxy-D-glucose: validation of method. *Ann. Neurol.* 6, 371–388 (1979).
28. Talairach, J. & Tournoux, P. in *Co-Planar Stereotaxic Atlas of the Human Brain* 1–122 (Theime, Stuttgart, 1988).
29. Fox, P. T., Perlmuter, J. S. & Raichle, M. E. A stereotactic method of anatomical localization for positron emission tomography. *J. Comput. Assist. Tomogr.* 9, 141–153 (1985).
30. Carmichael, S. T. & Price, J. L. Architectonic subdivision of the orbital and medial prefrontal cortex in the macaque monkey. *J. Comp. Neurol.* 346, 366–402 (1994).

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Relationship between subjective effects of cocaine and dopamine transporter occupancy

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Cocaine is believed to work by blocking the dopamine transporter (DAT) and thereby increasing the availability of free dopamine within the brain^{1–4}. Although this concept is central to current cocaine research and to treatment development, a direct relationship between DAT blockade and the subjective effects of cocaine has not been demonstrated in humans. We have used positron emission tomography to determine what level of DAT occupancy is required to produce a subjective 'high' in human volunteers who regularly abuse cocaine. We report here that intravenous cocaine at doses commonly abused by humans (0.3–0.6 mg kg^{−1}) blocked between 60 and 77% of DAT sites in these subjects. The magnitude of the self-reported high was correlated with the degree of DAT occupancy, and at least 47% of the transporters had to be blocked for subjects to perceive cocaine's effects. Furthermore, the time course for the high paralleled that of cocaine concentration within the striatum, a brain region implicated in the control of motivation and reward. This is the first demonstration in humans that the doses used by cocaine abusers lead to significant blockade of DAT, and that this blockade is associated with the subjective effects of cocaine. Although these findings provide justification to target the DAT for medication development they suggest that for drugs to be effective in blocking cocaine's effects they would have to be given at doses that achieve almost complete DAT occupancy.

Table 1 Measures for K_1 , distribution volume and $B_{\max}/K_d$ at baseline and after cocaine

Study	K_1		Distribution volume		$B_{\max}/K_d$
	ST	CB	ST	CB	
Baseline 1	0.51 ± 0.08	0.43 ± 0.10	5.00 ± 1.6	2.95 ± 0.9	0.69 ± 0.14
0.1 mg kg ⁻¹	0.44 ± 0.08	0.39 ± 0.09	3.93 ± 1.0	2.89 ± 0.7	0.36 ± 0.09
Baseline 2	0.50 ± 0.13	0.44 ± 0.11	5.11 ± 1.9	2.97 ± 0.7	0.69 ± 0.18
0.6 mg kg ⁻¹	0.54 ± 0.17	0.52 ± 0.17	3.73 ± 1.1	3.21 ± 0.7	0.16 ± 0.09
ANOVA	$F = 5, P < 0.01$		$F = 10, P < 0.0005$		$F = 41, P < 0.0001$

Plasma to brain transfer constant (K_1) and distribution volume (DV) for [¹¹C]cocaine in striatum (ST) and cerebellum (CB) and for striatal $B_{\max}/K_d$ ($(DV_{ST}/DV_{CB}) - 1$) for subjects receiving both the 0.1 and the 0.6 mg kg⁻¹ i.v. cocaine doses ($n = 8$). Comparisons correspond to a repeated measure ANOVA. NS, not significant.

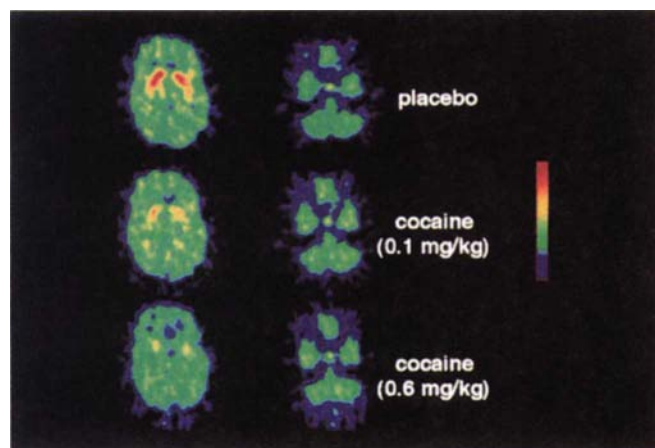


Figure 1 Distribution volume images at the level of the striatum and the cerebellum obtained with [¹¹C]cocaine for one of the subjects tested at baseline (placebo) and with 0.1 and 0.6 mg kg⁻¹ i.v. cocaine doses.

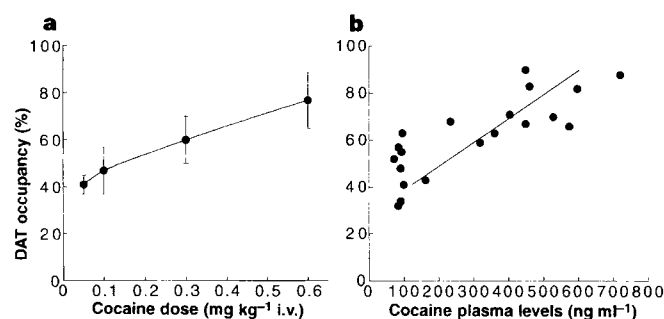


Figure 2 a, Correlation between dopamine transporter (DAT) occupancy by cocaine and doses of cocaine (mg kg⁻¹). Values correspond to means and standard deviations. **b**, Correlation between DAT occupancy and plasma concentration of cocaine (ng ml⁻¹).

This study investigates the relationship between DAT blockade and the reinforcing effects of cocaine in cocaine abusers. Occupation of DAT by cocaine was measured with positron emission tomography (PET) using [¹¹C]cocaine as a DAT ligand³. We selected [¹¹C]cocaine as a PET ligand because its fast pharmacokinetics are advantageous when attempting to investigate the effects of occupancies achieved by cocaine. Cocaine's fast association and dissociation from the DAT⁶ makes the occupancy hard to evaluate using ligands with slow dissociation constants under nonequilibrium conditions⁷. DAT occupancies were calculated using the ratio of the distribution volume of [¹¹C]cocaine in striatum to that in cerebellum, which corresponds to $B_{\max}/K_d + 1$ (ref. 8) where $B_{\max}$ is the total transporter concentration and K_d is the dissociation constant for cocaine. The reinforcing effects of cocaine, which in humans are generally accompanied by self-reports of euphoria or 'high', were assessed by asking participants to respond orally to four mood descriptors: 'high', 'rush', 'restlessness' and 'cocaine craving'¹⁰. We chose these self-reports of drug effects because they are reliable and consistent across studies and there is no other measure that is better in predicting administration of drugs in humans¹¹.

Cocaine significantly reduced the binding of [¹¹C]cocaine in striatum (Fig. 1) and the measures for DAT availability ($B_{\max}/K_d$) (Table 1). Estimations of DAT occupancies achieved with the different cocaine doses corresponded to 77% ± 10 (s.d.) for 0.6 mg kg⁻¹; 60% ± 10 for 0.3 mg kg⁻¹; 47% ± 10 for the 0.1 mg kg⁻¹ intravenous (i.v.) and 41% ± 3 for the 0.05 mg kg⁻¹ (Table 2). For subjects tested twice with the same cocaine dose the test-retest reproducibility of the occupancy measures was within 10% (test-retest occupancy measures for 0.3 mg kg⁻¹ doses were 59 ± 10 and 60 ± 10; and test-retest measures for the 0.05 mg kg⁻¹ i.v. dose were 42 ± 3 and 39 ± 4). DAT occupancies were signifi-

cantly correlated with the doses given ($r = 0.72$, d.f. 32; $P < 0.001$) and with the plasma concentration of cocaine for the 20 studies for which plasma cocaine concentrations were available ($r = 0.81$, d.f. 19; $P < 0.001$) (Fig. 2).

Cocaine significantly increased ratings of high and rush (0.6, 0.3 and 0.1 mg kg⁻¹) and craving (0.6 and 0.3 mg kg⁻¹). The 0.05 mg kg⁻¹ i.v. dose had no measurable effects (Table 2). Ratings of high were significantly correlated with DAT occupancy ($r = 0.55$, d.f. 32; $P < 0.001$) (Fig. 3). DAT occupancies > 60% were consistently associated with significantly increased ratings of high relative to baseline. Ratings of rush were also significantly correlated with DAT occupancy ($r = 0.54$, d.f. 32; $P < 0.001$), but this was not true for either restlessness or craving.

To evaluate the temporal relationship between the drug-induced high and the kinetics of cocaine in brain we plotted the time activity curves for [¹¹C]cocaine in striatum and the curve for the high for one of the subjects (Fig. 4). There was a very good correspondence between the temporal course for cocaine-induced high and the pharmacokinetics of [¹¹C]cocaine in striatum at the time of peak behavioural effects, which is when peak concentration of cocaine in brain occurs. After peak effects, self-reports for the high declined faster than the rate of clearance of cocaine from brain. Although we had previously shown this temporal correspondence using behavioural data taken from the literature⁵, these results confirm the temporal correspondence of these two events measured simultaneously in the same individual.

To the extent that increases in self-report measures such as high are predictive of reinforcing effects¹², our findings of significant correlations between the level of DAT occupancy and ratings of high, and between the temporal course for the high and the kinetics of cocaine in striatum corroborate in humans an association between DAT blockade and cocaine's mood-enhancing effects.

Table 2 Behavioural changes induced by cocaine and plasma cocaine concentration

Intervention	Number of studies	High (0–10)	Rush (0–10)	Craving (0–10)	Restlessness (0–10)	Plasma (ng ml ⁻¹)
Placebo	33	1 ± 2	1 ± 2	2 ± 3	3 ± 4	0
0.05 mg kg ⁻¹ i.v.	4	2 ± 1	1 ± 1	3 ± 3	2 ± 2	NA
0.1 mg kg ⁻¹ i.v.	10	4 ± 3*	4 ± 3*	4 ± 1	5 ± 3	97 ± 25
0.3 mg kg ⁻¹ i.v.	11	5 ± 3***	7 ± 4***	5 ± 3*	4 ± 3	300 ± 60
0.6 mg kg ⁻¹ i.v.	8	8 ± 2***	8 ± 2***	7 ± 4**	2 ± 2	520 ± 100

Cocaine-induced behavioural changes (cocaine/placebo) and plasma cocaine concentration at 8 min after its administration. Behavioural scores correspond to peak effects. Comparison with placebo (ANOVA): **P* < 0.01, ***P* < 0.01, ****P* < 0.001. NA, not available.

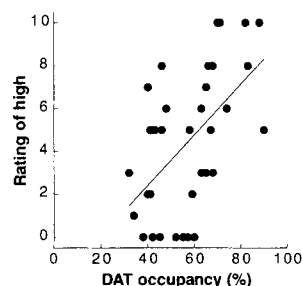


Figure 3 Correlation between dopamine transporter (DAT) occupancy by cocaine and subjects' reports of high.

The very fast uptake of cocaine in brain (Fig. 4) in addition to its high brain uptake (10% of the injected dose)⁵ explain the high levels of DAT occupancy achieved with cocaine at the doses commonly used by cocaine addicts. Also, one can predict that the fast uptake and binding of cocaine in brain (Fig. 4) generates a fast blockade of DAT with a consequent fast change in dopamine concentration¹³. Because the rapidity of a drug's actions in brain are crucial in its reinforcing effects¹⁴ this could explain why cocaine is more reinforcing than other drugs with higher affinity for the DAT but with slower pharmacokinetics¹⁵. Thus, the highly reinforcing effects of cocaine are likely to be due not only to its ability to block the DAT but to its ability to block DAT at a very fast rate¹⁵.

The intravenous doses commonly abused by humans, which range between 0.35 mg kg⁻¹ and 0.71 mg kg⁻¹ (25–50 mg)¹⁶ are likely to inhibit on average 65 and 80% of the DAT, respectively. This indicates that although cocaine at doses used by humans induces significant DAT occupancy it does not saturate the DAT. These findings have important implications for the development of medications for cocaine treatment, because a major strategy is the development of compounds that interfere with cocaine binding to the DAT¹⁷. Thus, the findings from this study suggest that a 'cocaine substitute' medication would be effective only if given at doses that achieve almost complete DAT blockade.

A limitation of this study is that measurements were made in the dorsal striatum and not in the nucleus accumbens, the structure associated with drug reinforcement in laboratory animals^{18,19}; however, DAT occupancy by cocaine should not differ appreciably between these brain regions²⁰. Also, the occupancy measures may underestimate maximal DAT blockade because cocaine reaches peak plasma and brain concentrations 4–6 min after its intravenous administration^{5,21}, whereas our PET measures are weighted to the initial 20 min of the 54 min imaging period⁵. However, these estimates correspond well to those obtained from simulation studies done to calculate 'peak occupancies'²².

This study provides the first documentation, to our knowledge, of a significant relationship between DAT blockade by cocaine and ratings of high and rush in humans. It also shows that DAT blockade greater than 47% is required for cocaine to be subjectively perceived

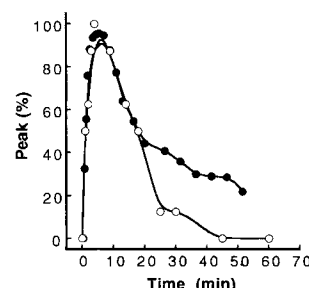


Figure 4 Time activity curves for [¹¹C]cocaine in striatum (filled circles) alongside the temporal course for cocaine-induced high (empty circles) for one of the subjects. For comparison purposes values are expressed as per cent from peak. Notice the good correspondence between the kinetics of [¹¹C]cocaine in striatum and the duration of cocaine-induced high for the initial uptake and peak concentration of cocaine in striatum. The high dissipates more rapidly than the levels of [¹¹C]cocaine.

as reinforcing in experienced cocaine users and corroborates a temporal correspondence between the duration of the high induced by cocaine and its kinetics in striatum. These results consolidate the role of DAT blockade as a crucial mechanism in the reinforcing properties of cocaine in humans. □

Methods

Subjects. The subjects were 17 current cocaine users (12 male and 5 female, age 35 ± 5 years) selected from a pool of cocaine abusers who responded to an advertisement. Subjects were initially screened by phone and then evaluated as outpatients at Columbia University to ensure inclusion criteria ((1) DSM IV diagnostic criteria for active cocaine dependence; (2) continuous use of cocaine for at least the prior 6 months with claimed cocaine use of at least three grams a week; (3) use of cocaine as smoked free-base and/or intravenously; (4) lack of current or past psychiatric and/or neurological disease (other than cocaine dependence); (5) no significant medical illness with special care for any evidence for cardiac pathology) and to obtain the physical examination and the laboratory tests. Cocaine abusers had a history of abusing 4 ± 3 g cocaine per week for 10 ± 5 years and their last use of cocaine was 5 ± 7 days before the scan. Pre-scan tests ensured the absence of any psychoactive drug use except cocaine. Informed consents were obtained from the subjects after carefully explaining the procedures.

Scans. PET studies were carried out with a CTI 931 tomograph (6 × 6 × 6.5 mm full width half maximum) using [¹¹C]cocaine as a DAT ligand^{5,23}. Methods for positioning subjects in the tomograph, catheterizations, transmission scans, blood sampling and blood analysis have been published⁵. Briefly, emission scans were started immediately after injection of 4–8 mCi of [¹¹C] cocaine (specific activity > 0.2 Ci μmol⁻¹ at time of injection). A series of 20 emission scans were obtained from time of injection up to 54 min. Arterial sampling was used to quantify total carbon-11 and unchanged [¹¹C]cocaine in plasma as described⁵. Each subject was scanned 4 times with [¹¹C]cocaine over a 2-day period. The initial scan on each day was done with placebo (3 cm³ saline) coadministered with a tracer quantity of [¹¹C]cocaine and was used as baseline; the second scan on each day was performed 2 h after the first one and was done with active doses of cocaine coadministered with [¹¹C]cocaine. In eight of the subjects the doses of coadministered cocaine in these two scans were 0.1 and 0.6 mg kg⁻¹ i.v.; and in one subject they were 0.1 and 0.3 mg kg⁻¹. Additionally, to assess test–retest reproducibility, the DAT occupancies achieved with

0.3 mg kg⁻¹ i.v. cocaine were measured twice in 5 of the subjects and the occupancies achieved with 0.05 mg kg⁻¹ i.v. cocaine were measured twice in 2 of the subjects. One subject was scanned only twice (baseline, 0.1 mg kg⁻¹ i.v.). Venous blood was drawn for the determination of cocaine and cocaine metabolites 8, 15, 30 and 45 min after its administration. Concentration of cocaine in plasma was quantified using capillary GC/mass spectrometry. Because of relocation of the National Psychopharmacology Laboratory (NPL) measurements were only available for 20 of the 33 cocaine interventions.

Drug effect ratings. In order to simulate an analogue rating scale participants were instructed to respond to each descriptor using a whole number between 1 and 10 for the self-report of the high, rush, restlessness and cocaine craving. High was defined as euphoria, rush as the sensation of the drug in the body, restlessness as desire to move and cocaine craving as desire for cocaine. Ratings were obtained every minute for the first 20 min and then at 10-min intervals for the next 40 min as previously described¹⁰. Heart rate and blood pressure were monitored continuously.

Image and data analysis. Regions of interest in striatum and cerebellum were drawn directly on average [¹¹C]cocaine image (images obtained between 10 and 54 min) as previously described⁵. These regions were then projected onto the dynamic images to generate time activity curves for striatum and for cerebellum. These time activity curves for tissue concentration along with the time activity curves for unchanged tracer in plasma were used to calculate the distribution volume (DV) in striatum and cerebellum using a graphic analysis technique for reversible systems (Logan Plots)²⁴. The ratio of the DV in striatum to that in cerebellum, which corresponds to $B_{\max}/K_d + 1$ and is insensitive to changes in cerebral blood flow, was our measure of DAT availability⁸. DAT occupancies were calculated as: $((B_{\max}/K_{d\text{placebo}} - B_{\max}/K_{d\text{cocaine}}) \times 100$. Differences in DAT occupancy at baseline and after cocaine injection were tested with repeated measures ANOVA. Pearson product moment correlations analyses were calculated for the estimates of DAT occupancy and the subjective effect changes (cocaine/placebo) and also between DAT occupancy and the concentration of cocaine in plasma.

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- Ritz, M. C., Lamb, R. J., Goldberg, S. R. & Kuhar, M. J. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* **237**, 1219–1223 (1987).
- Koob, G. F. & Bloom, F. E. Cellular and molecular mechanism of drug dependence. *Science* **242**, 715–723 (1988).
- Madras, B. K., Fahey, M. A., Bergman, J., Canfield, D. R. & Speelman, R. D. Effects of cocaine and related drugs in non-human primates. I. [³H]cocaine binding sites in caudate-putamen. *J. Pharmacol. Exp. Ther.* **251**, 131–141 (1989).
- Giros, B., Jaber, M., Jones, S. R., Wightman, R. M. & Caron, M. G. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606–612 (1996).
- Fowler, J. et al. Mapping cocaine-binding sites in human and baboon brain *in vivo*. *Synapse* **4**, 371–377 (1989).
- Pogun, S., Scheffel, U. & Kuhar, M. J. Cocaine displaces 3H-WIN 35,248 binding to dopamine uptake sites *in vivo* more rapidly than mazindol or GBR 12,909. *Eur. J. Pharmacol.* **198**, 203–205 (1991).
- Gatley, S. J. et al. Displacement of RTI-55 from the dopamine transporter by cocaine. *Eur. J. Pharmacol.* **296**, 145–151 (1996).
- Logan, J. et al. Effects of blood flow on [¹¹C] raclopride binding in the brain: model simulations and kinetic analysis of PET data. *J. Cereb. Blood Flow Metab.* **14**, 995–1010 (1994).
- Nestler, E. J. Molecular mechanisms of drug addiction. *J. Neurosci.* **12**, 2439–2450 (1992).
- Volkow, N. D. et al. Temporal relationships between the pharmacokinetics of methylphenidate in the human brain and its behavioral and cardiovascular effects. *Psychopharmacology* **123**, 26–33 (1996).
- Fischman, M. W. & Foltin, R. W. Utility of subjective-effects measurements in assessing abuse liability of drugs in humans. *Br. J. Addiction* **86**, 1563–1570 (1991).
- Fischman, M. W. in *Testing for Abuse Liability of Drugs in Humans*, NIDA Research Monographs Vol. 92 (eds Fischman, M. W. & Mello, N. H.) 211–230 (Government Printing Office, Washington DC, 1989).
- Pettit, H. O. & Justice, J. B. Effect of dose in cocaine self-administration behavior and dopamine levels in the nucleus accumbens. *Brain Res.* **539**, 94–102 (1991).
- Balster, R. L. & Schuster, C. R. Fixed interval schedule of cocaine reinforcement: effect of dose and infusion duration. *J. Exp. Anal. Behav.* **20**, 119–129 (1973).
- Stathis, M. et al. Rate of binding of various inhibitors at the dopamine transporter *in vivo*. *Psychopharmacology* **119**, 376–384 (1995).
- Verbey, K. & Gold, M. S. From cocaine leaves to crack: the effects of dose and routes of administration in abuse liability. *Psychiatr. Ann.* **18**, 513–520 (1988).
- Rothman, R. B. High affinity dopamine reuptake inhibitors as potential cocaine antagonists: a strategy for drug development. *Life Sci.* **46**, 17–21 (1990).
- Pettit, H. O., Ettenberg, A., Bloom, F. E. & Koob, G. F. Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology* **84**, 167–173 (1984).
- Pontieri, F. E., Tanda, G., Orzi, F. & Di Chiara, G. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* **382**, 255–257 (1996).
- Izenwasser, S., Werling, L. L. & Cox, B. M. Comparison of the effects of cocaine and other inhibitors of dopamine uptake in rat striatum, nucleus accumbens, olfactory tubercle, and medial prefrontal cortex. *Brain Res.* **520**, 303–309 (1990).
- Javadi, J. I., Fischman, M. W., Schuster, C. R., Dekirmenjian, H. & Davis, J. M. Cocaine plasma concentration: relation to physiological and subjective effects in human. *Science* **202**, 227–228 (1978).
- Gatley, S. J. et al. A model for estimating dopamine transporter occupancy and subsequent increases in

synaptic dopamine using positron emission tomography and carbon-11 labeled cocaine. *Biochem. Pharmacol.* **51**, 43–52 (1997).

23. Volkow, N. D. et al. Carbon-11-cocaine binding compared at sub-pharmacological and pharmacological doses: a PET study. *J. Nucl. Med.* **36**, 1289–1297 (1995).

24. Logan, J. et al. Graphical analysis of reversible radioligand binding from time-activity measurements applied to [¹¹C-methyl]-(-)-cocaine PET studies in human subjects. *J. Cereb. Blood Flow Metab.* **10**, 740–747 (1990).

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Decreased striatal dopaminergic responsiveness in detoxified cocaine-dependent subjects

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Cocaine blocks the reuptake of dopamine, a neurotransmitter involved in the control of movement, cognition, motivation and reward. This leads to an increase in extracellular dopamine; the reinforcing effect of cocaine is associated with elevated dopamine levels in the nucleus accumbens^{1,2}. But addiction to cocaine involves other effects, such as craving, loss of control and compulsive drug intake; the role of the dopamine system in these effects is less well-understood. We therefore used positron emission tomography (PET) to compare the responses of cocaine addicts and normal controls to intravenous methylphenidate, a drug that, like cocaine, causes an increase in synaptic dopamine³. Addicts showed reduced dopamine release in the striatum, the brain region where the nucleus accumbens is located, and also had a reduced 'high' relative to controls. In contrast, addicts showed an increased response to methylphenidate in the thalamus (a region that conveys sensory input to the cortex). This thalamic response was associated with cocaine craving and was not seen in control subjects. Thus, our findings challenge the notion that addiction involves an enhanced striatal dopamine response to cocaine and/or an enhanced induction of euphoria. Moreover, they suggest a participation of thalamic dopamine pathways in cocaine addiction, a possibility that merits further investigation. It has been hypothesized that cocaine addiction could result from decreased function of dopamine in the brain⁴. Hence we compared the function of the brain dopamine system of 20 cocaine-dependent subjects with 23 controls using positron emission tomography (PET) and [¹¹C]raclopride, a dopamine D2 receptor ligand sensitive to competition with endogenous dopamine⁵, by measuring the relative changes in extracellular dopamine induced by intravenous methylphenidate (MP)⁶. MP, like cocaine, increases synaptic dopamine by inhibiting dopamine reuptake³, it has equivalent reinforcing effects to those of cocaine⁷, and its intravenous administration induces a 'high' similar to that of cocaine⁸. Because [¹¹C]raclopride binding is highly reproducible⁹, differences in binding between placebo and MP predominantly reflect MP-induced changes in extracellular dopamine^{10,11}. Dopamine responses to MP (0.5 mg kg⁻¹ intravenously (i.v.)) were measured

in striatum and thalamus¹², brain regions where binding of D2 PET radioligands is inhibited by D2 antagonists¹³.

Parallel assessment of the behavioural effects of MP revealed significant differences between the groups; whereas MP-induced 'high' and 'restlessness' was more intense in controls MP-induced 'cocaine craving' was more intense in cocaine-dependent subjects (Table 1). There were no differences between the groups in MP-induced cardiovascular changes (not shown), or in plasma MP concentration (controls, 119 s.d. 36 ng ml⁻¹; dependent subjects, 107 s.d. 36 ng ml⁻¹).

Figure 1 shows representative [¹¹C]raclopride images at the level of striatum obtained after placebo and after MP in a control and in a cocaine-dependent subject. Although MP did not affect the transport of [¹¹C]raclopride from blood to brain (rate, K_1) for any of the brain regions it induced a significant reduction in the distribution volume (DV) in all brain regions as well as in the estimates for dopamine D2 receptor availability (B_{max}/K_d) in striatum (Table 2). The DV in cerebellum, which is used to normalize the receptor measures for global changes in DV, was decreased by MP. The magnitude of this decrease, which reflects a global change in the relative distribution of raclopride between tissue and plasma with MP¹⁴, did not differ between groups (ANOVA diagnosis by drug interaction effect $F = 0.19$; d.f. 1,41; $P = 0.67$). In contrast, MP-induced decreases in DV in striatum were significantly larger in controls than in dependent subjects (interaction effect $F = 5.6$, $P = 0.02$). Similarly, MP-induced changes in B_{max}/K_d in striatum were significantly larger in controls (21 s.d. 13% change from baseline) than in cocaine-dependent subjects (9 s.d. 13%) (interaction effect $F = 9.3$, $P < 0.004$) (Fig. 2). There were also significant differences between the groups in the response to MP in thalamus; the DV decreases in thalamus were significantly larger in the cocaine-dependent subjects ($F = 5.4$, $P = 0.03$) and in cocaine-dependent subjects, but not in controls, MP decreased B_{max}/K_d values in thalamus (29 s.d. 35%) (interaction effect $F = 11.0$, $P < 0.002$) (Fig. 2).

In striatum, MP-induced changes in B_{max}/K_d were significantly correlated with MP-induced changes in self reports of 'restlessness' (correlation for the two groups: $r = 0.49$, d.f. 42; $P < 0.002$; correlation for cocaine-dependent subjects only: $r = 0.52$, d.f. 19; $P < 0.05$). In thalamus, MP-induced changes in B_{max}/K_d were significantly correlated with MP-induced changes in 'cocaine craving' (correlation for the two groups: $r = 0.57$, d.f. 42; $P < 0.0001$; correlation for cocaine-dependent subjects only: $r = 0.61$, d.f. 19;

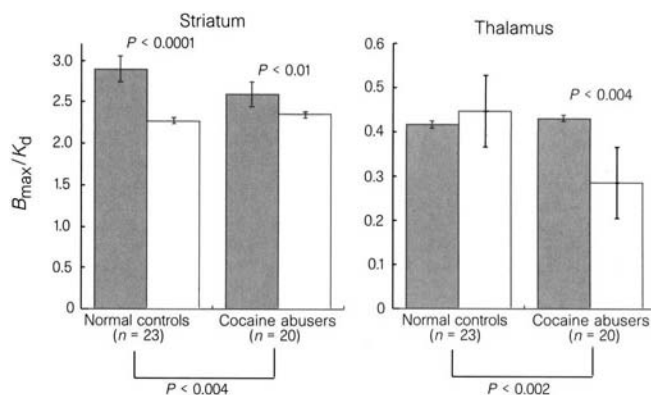


Figure 2 Mean and standard error for the B_{max}/K_d estimates in striatum and in thalamus after placebo (filled bar) and after methylphenidate (MP) administration (empty bar) in normals and in cocaine-dependent subjects. In striatum, results for the ANOVA reveal a significant drug effect ($F = 51$, d.f. 1; $P < 0.0001$) as well as a significant drug by diagnosis interaction effect ($F = 9.3$, d.f. 1,41; $P < 0.004$). Cocaine-dependent subject's response to MP was significantly smaller than that of controls (baseline-MP). In thalamus, MP significantly decrease B_{max}/K_d only in cocaine-dependent subjects ($P < 0.004$). At baseline, B_{max}/K_d in striatum was significantly lower in cocaine-dependent subjects than in controls ($P < 0.01$).

$P < 0.005$) (Fig. 3). B_{max}/K_d changes were not correlated with the high or with plasma MP concentration (not shown).

Because MP increases dopamine by blocking dopamine transporters, the blunted response to MP in striatum may reflect decreased outflow of dopamine in the baseline state as well as changes in dopamine in anticipation of receiving a psychostimulant drug. These results are compatible with a decrease in striatal dopamine brain function in cocaine-dependent subjects. Decreased striatal dopamine responsiveness would make cocaine, in principle, less reinforcing because it would be the equivalent of administering smaller doses. One could also predict that decreased dopamine responsiveness would result in a blunted response to behaviours resulting from increases in striatal dopamine. In this respect MP-induced restlessness was more intense in normals than in dependent subjects and was correlated with changes in striatal dopamine. Similarly the high from MP in the dependent subjects was of a lower intensity than in controls and although it was not associated

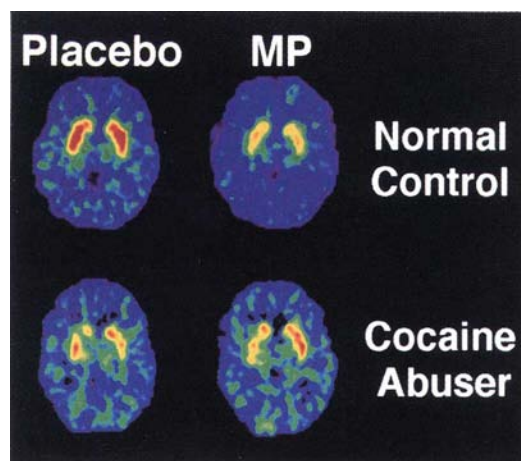


Figure 1 Distribution volume images of [¹¹C]raclopride at the level of the striatum in a normal control and in a cocaine-dependent subject tested after placebo (baseline) and after methylphenidate (MP) administration. Baseline binding for [¹¹C]raclopride in striatum and the reductions in striatal binding with MP were lower in the cocaine-dependent subject than in the control.

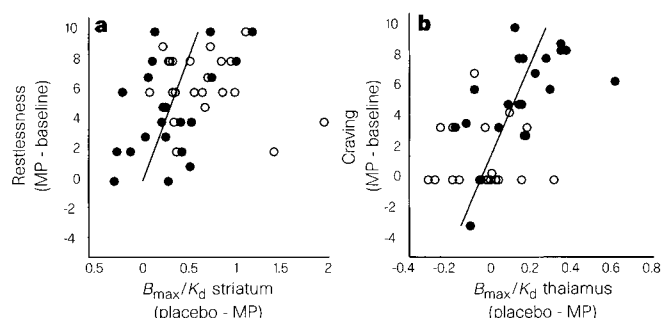


Figure 3 **a**, Regression slopes for the correlation between methylphenidate (MP)-induced changes in dopamine D2 receptor availability in striatum and MP-induced changes in self-reports of restlessness. **b**, Regression slopes for the correlation between MP-induced changes in dopamine receptor availability in thalamus and MP-induced changes in self-reports of cocaine craving. Normal controls are shown as empty circles and cocaine-dependent subjects as filled circles.

Table 1 Mean and standard deviations ($\pm$) for self reports of drug-induced (placebo or methylphenidate) behavioural effects in controls and in cocaine-dependent subjects

	Normal controls		Cocaine dependent		MP effect
	Placebo	MP	Placebo	MP	<i>P</i>
Alert	7 $\pm$ 1	9 $\pm$ 1	8 $\pm$ 1	9 $\pm$ 2	NS
Anxiety	2 $\pm$ 2	4 $\pm$ 3	4 $\pm$ 3	6 $\pm$ 4	NS
Distrustful	0	1 $\pm$ 2	1 $\pm$ 1	2 $\pm$ 3	NS
High*	1 $\pm$ 1	6 $\pm$ 3	0	4 $\pm$ 3	<i>P</i> < 0.005
Restlessness**	2 $\pm$ 2	7 $\pm$ 2	2 $\pm$ 2	5 $\pm$ 4	<i>P</i> < 0.0001
Craving***	0	1 $\pm$ 2	3 $\pm$ 3	8 $\pm$ 3	<i>P</i> < 0.001

Self reports were recorded 27 min after administration of placebo or of methylphenidate (MP). MP significantly increased self reports for high, restlessness and cocaine craving. Behavioural responses to MP were significantly larger in controls for restlessness and high whereas for cocaine craving responses were larger in the cocaine-dependent subjects. ANOVA interaction effect (diagnosis $\times$ drug): **P* < 0.05, ***P* < 0.005, ****P* < 0.001. NS, not significant.

with changes in striatal dopamine, this could have been due to the fact that measurements were done in dorsal striatum and not in nucleus accumbens, the brain structure associated with drug reinforcement in laboratory animals¹⁵, and that measurements were recorded at 27 min when peak effects for the high had subsided. In laboratory animals, prior exposure to psychostimulants results in accentuated drug responses and this phenomenon of sensitization, which in some studies but not in others has been associated with enhanced dopamine responses, has been implicated in cocaine addiction¹⁶. The blunted response to MP in cocaine-dependent subjects does not support an enhancement of dopamine release and/or of the reinforcing effects of cocaine as one of the mechanisms underlying human addiction. The discrepancies with some of the laboratory animal data are likely to reflect differences between species, patterns of psychostimulant administration¹⁶, and/or conditions of drug administration¹⁷.

Addicted subjects were distinguished from controls by the cocaine craving induced by MP. Under appropriate conditions, the craving would have most likely led to compulsive cocaine intake ('bingeing'). The abnormal activation of the thalamus in dependent subjects, but not in controls, and its significant association with MP-induced cocaine craving suggests that abnormal activation of thalamic dopamine pathways may participate in the expression of this unique behaviour of the addicted subject. The thalamus has a relatively high uptake of cocaine^{18,19} and it is sensitive to D2 receptor-mediated effects of cocaine²⁰. Although current PET scanners lack the spatial resolution to distinguish individual thalamic nuclei, one could speculate that MP-induced dopamine changes in the dependent subjects involve the mediodorsal nucleus (MD), which receives dopamine projections²¹ and is a relay between accumbens and orbitofrontal cortex²². MD lesions disrupt conditioned reinforcement behaviours²³. Because pathology in orbitofrontal cortex is associated with the emergence of repetitive behaviours in laboratory animals²⁴, and with compulsive disorders in humans²⁵, it is possible that abnormal MD activation in cocaine-dependent subjects activates the orbitofrontal cortex, which enables the compulsive drug intake. In this respect it is interesting to note that decreases in D2 receptors in cocaine-dependent subjects are associated with metabolic abnormalities in orbitofrontal cortex²⁶ and that the striato-thalamic-orbitofrontal circuit has been postulated as a common mechanism underlying drug and alcohol addiction^{26,27}. However, because the specific to nonspecific binding ratio of [¹¹C]raclopride in thalamus is quite low and MP decreased the nonspecific binding of raclopride (DV in cerebellum) we cannot exclude the possibility that MP-induced changes in thalamus in cocaine-dependent subjects reflects nonspecific effects and further studies are required to replicate these findings.

Baseline striatal D2 $B_{\max}/K_d$ values were lower in cocaine-dependent subjects (2.59 s.d. 0.3) than in controls (2.90 s.d. 0.3) (*P* < 0.01) and

Table 2

	Normal controls		Cocaine dependent		MP effect
	Placebo	MP	Placebo	MP	<i>P</i>
K_1					
Striatum	0.10 $\pm$ 0.02	0.10 $\pm$ 0.02	0.10 $\pm$ 0.02	0.10 $\pm$ 0.02	NS
Thalamus	0.09 $\pm$ 0.08	0.10 $\pm$ 0.03	0.13 $\pm$ 0.07	0.17 $\pm$ 0.13	NS
Cerebellum	0.06 $\pm$ 0.02	0.07 $\pm$ 0.02	0.08 $\pm$ 0.03	0.08 $\pm$ 0.03	NS
DV					
Striatum*	1.62 $\pm$ 0.3	1.24 $\pm$ 0.2**	1.62 $\pm$ 0.2	1.43 $\pm$ 0.2*	0.0001
Thalamus*	0.59 $\pm$ 0.08	0.55 $\pm$ 0.09	0.65 $\pm$ 0.10	0.55 $\pm$ 0.11	0.0001
Cerebellum	0.42 $\pm$ 0.06	0.38 $\pm$ 0.08	0.45 $\pm$ 0.06	0.43 $\pm$ 0.06	0.001
$B_{\max}/K_d$					
Striatum**	2.90 $\pm$ 0.34	2.27 $\pm$ 0.37	2.59 $\pm$ 0.34	2.35 $\pm$ 0.41	0.0001
Thalamus**	0.42 $\pm$ 0.10	0.45 $\pm$ 0.15	0.43 $\pm$ 0.15	0.29 $\pm$ 0.15	0.04

MP did not change K_1 but it decreased the DV and the $B_{\max}/K_d$. MP-induced changes in DV and $B_{\max}/K_d$ in striatum were significantly smaller in cocaine-dependent subjects whereas MP-induced changes in DV and $B_{\max}/K_d$ in thalamus were significantly larger in cocaine-dependent subjects than in controls. ANOVA interaction effect (diagnosis $\times$ drug): **P* < 0.02, ***P* < 0.005. Striatal $B_{\max}/K_d$ measures for the placebo condition were significantly lower (*P* < 0.01) in cocaine-dependent subjects than in controls.

agree with previous findings of D2 receptor reductions in cocaine-dependent subjects²⁶. Striatal D2 receptor measurements with PET predominantly reflect postsynaptic receptors²⁸ and thus indicate involvement of GABA and/or acetylcholine cells. The decreased response to MP in striatum, on the other hand, indicates that there are also functional changes in DA cells in cocaine-addicted subjects.

Although unlikely, we cannot exclude the possibility that the decreased response to MP in cocaine-dependent subjects could also result from affinity changes in dopamine receptors and/or from changes in dopamine transporters. Although raclopride binds both to D2 and to D3 receptors, the striatal concentration of D3 receptors is very low so that the PET measurements mainly reflect binding to D2 receptors²⁸. Also, although this study evaluated subjects 3–6 weeks after drug discontinuation and the findings may not generalize to periods of more protracted withdrawal, it represents a period when addictive behaviours rapidly emerge²⁹ and in this respect directly pertains to the investigation of cocaine addiction.

This study provides evidence of decreased striatal dopamine brain function in cocaine-dependent subjects. It also shows an abnormal response in thalamus in cocaine-dependent subjects which was associated with cocaine craving. Further studies are required to determine if MP-induced changes in thalamic raclopride binding in cocaine-dependent subjects reflect competition with dopamine and to assess its role in the loss of control and the compulsive drug intake in the cocaine addict. □

Methods

Subjects. Cocaine-dependent subjects were 20 male right-handed subjects (age 36 s.d. 4 years) studied 3–6 weeks after drug discontinuation. Subjects were inpatients who fulfilled DSM IV diagnostic criteria for cocaine dependence and had used cocaine (free-base or crack) for at least the prior 6 months (at least 'four grams' a week) but were otherwise medically healthy. Exclusion criteria were current or past psychiatric (other than cocaine dependence), neurological, cardiovascular or endocrinological disease, dependence on any substance other than cocaine, nicotine or caffeine and more than moderate (12 ounces per week) use of ethanol. Controls were 23 male right-handed healthy subjects (age 34 s.d. 7 years) who were screened for a lack of history of drug or alcohol abuse (excluding nicotine/caffeine). Exclusion criteria were otherwise as for cocaine-dependent subjects. None of the subjects was taking medications at the time of the study. Toxicological drug screens were performed before each PET scan. Informed consent was obtained for all subjects after procedures were explained.

Scans. Subjects had two scans done with [¹¹C]raclopride, the first scan after placebo and the second after 0.5 mg kg⁻¹ i.v. MP, performed on separate days at the same time of day. Subjects were blind to whether placebo or MP was administered, which were injected 6–9 min before [¹¹C]raclopride. Scans were done using a CTI 931 tomography (6 $\times$ 6 $\times$ 6.5 mm full width half maximum) after i.v. injection of 4–10 mCi of [¹¹C]raclopride (specific activity 0.5–1.5 Ci μ M⁻¹ at end of bombardment) as described⁹.

Drug effect ratings. Behavioural effects were evaluated using analogue scales that assessed self reports of alertness, anxiety, distrustfulness, high, restlessness and cocaine craving from 0 (felt nothing) to 10 (felt intensely)⁸ recorded 5 min before and 27 min after MP or placebo. Cocaine craving was scored both as the desire for cocaine and the perception of loss of control for drug intake if the drug were to be available. Vital signs were obtained periodically.

Image analysis. Regions of interest (ROI) were outlined for striatum (ST), thalamus (TH) and cerebellum (CB) by an investigator blind to the subject's diagnosis using procedures previously described⁹. Briefly, ROI were initially outlined on the individual's summed baseline [¹¹C]raclopride image (images obtained between 15 and 54 min) and were then projected into the subject's corresponding coregistered MRI to ensure adequate anatomical localization. ROI were then projected into the dynamic [¹¹C]raclopride images for the baseline and MP studies to generate time activity curves for ST, TH and CB. These time activity curves for tissue concentration along with the time activity curves for unchanged tracer in plasma were used to calculate [¹¹C]raclopride's transfer constant from plasma to brain (K_1) and the distribution volumes (DV), which correspond to the equilibrium measurement of the ratio of tissue concentration to plasma concentration in ST, TH and CB using a graphical analysis technique for reversible systems¹⁰. The ratios of DV in ST and DV in TH to those of DV in CB correspond to $(B_{\max}/K_d) + 1$ and are insensitive to changes in cerebral blood flow¹⁴. The response to MP was quantified as the difference in $B_{\max}/K_d$ between placebo and MP.

$B_{\max}/K_d$ can be related to the DV as follows

$$DV = (K_1/k_2)(1 + NS + (B_{\max} - RL)/K_d)$$

K_1 and k_2 are plasma to tissue and tissue to plasma transport constants, respectively. $B_{\max}$ is the total D2 receptor concentration and RL is concentration of receptors occupied with endogenous dopamine. NS is the ratio of binding constants for nonspecific binding, which is considered to be essentially instantaneous (compared to receptor binding) and therefore in an equilibrium state. In a non-receptor region the DV is given by

$$DV = K_1/k_2(1 + NS)$$

Assuming that the ratio K_1/k_2 is the same for receptor and nonreceptor regions, the ratio of DV for ST to CB is

$$DV(ST)/DV(CB) = (B_{\max} - RL)/(K_d(1 + NS)) + 1$$

Although the DV is an equilibrium quantity it can be easily determined from nonequilibrium data by graphical analysis. The free receptor concentration ($B_{\max} - RL$) is therefore a linear function of the DV ratio. We have designated $B_{\max}/K_d$ as the quantity $(B_{\max} - RL)/K_d$ where we have replaced $K_d(1 + NS)$ with K_d , which is the K_d that comes out of PET measurements without having to measure the quantity NS separately.

Data analysis. Differences in $B_{\max}/K_d$ between groups were compared using ANOVA. Pearson correlations were used to assess the relationship between MP-induced changes in $B_{\max}/K_d$ and its behavioural effects. Differences in the behavioural and cardiovascular response to MP between groups were tested with nonparametric tests (Mann-Whitney *U*-test) using score differences between placebo and MP. Values of $P < 0.01$ were considered significant and P values between 0.01 and 0.05 are reported as trends.

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- Koob, G. F. & Bloom, F. E. Cellular and molecular mechanism of drug dependence. *Science* **242**, 715–723 (1988).
- Di Chiara, G. & Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl Acad. Sci. USA* **85**, 5274–5278 (1988).
- Volkow, N. D. *et al.* Is methylphenidate like cocaine? Studies on their pharmacokinetics and distribution in human brain. *Arch. Gen. Psychiatry* **52**, 456–463 (1995).
- Dackis, C. A. & Gold, M. S. New concepts in cocaine addiction: the dopamine depletion hypothesis. *Neurosci. Biobehav.* **9**, 469–477 (1985).
- Seeman, P., Guan, H. C. & Niznik, H. B. Endogenous dopamine lowers the dopamine D2 receptor density as measured by 3H raclopride: implications for positron emission tomography of the human brain. *Synapse* **3**, 96–97 (1996).
- Volkow, N. D. *et al.* Imaging endogenous dopamine competition with [¹¹C]raclopride in the human brain. *Synapse* **16**, 255–262 (1994).
- Bergman, J., Madras, B. K., Johnson, S. E. & Spealman, R. D. Effects of cocaine and related drugs in nonhuman primates: III. Self administration by squirrel monkeys. *J. Pharmacol. Exp. Ther.* **251**, 150–155 (1989).
- Wang, G.-J. *et al.* Behavioral and cardiovascular effects of intravenous methylphenidate in normal subjects and cocaine abusers. *Eur. Addiction Res.* **3**, 49–54 (1997).
- Volkow, N. D. *et al.* Reproducibility of repeated measures of [¹¹C] raclopride binding in the human brain. *J. Nucl. Med.* **34**, 609–613 (1993).

- Dewey, S. L. *et al.* Striatal binding of the PET ligand [¹¹C]-raclopride is altered by drugs that modify synaptic dopamine levels. *Synapse* **13**, 350–356 (1993).
- Gifford, A., Gatley, S. J. & Ashby, C. R. Endogenously released dopamine inhibits the binding of dopaminergic PET and SPECT ligands in superfused rat striatal slices. *Synapse* **22**, 232–238 (1996).
- Wang, G.-J. *et al.* Comparison of two PET radioligands for imaging extrastriatal dopamine receptors in human brain. *Synapse* **15**, 246–249 (1993).
- Youssef, K. *et al.* Inhibition of extrastriatal dopamine receptors by haloperidol. *Synapse* **19**, 14–17 (1995).
- Logan, J. *et al.* Effects of blood flow on [¹¹C]raclopride binding in the brain: model simulations and kinetic analysis of PET data. *J. Cereb. Blood Flow Metab.* **14**, 995–1010 (1994).
- Pontieri, F. E., Tanda, G., Orzi, F. & Di Chiara, G. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* **382**, 255–257 (1996).
- Robinson, T. E. & Berridge, K. C. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res. Rev.* **18**, 247–291 (1993).
- Brown, E. E., Robertson, G. S. & Fibiger, H. C. Evidence for conditioned neuronal activation following exposure to a cocaine paired environment: role of forebrain limbic structures. *J. Neurosci.* **12**, 4112–4121 (1992).
- Wang, G.-J. *et al.* Comparison of two PET radioligands for imaging extrastriatal dopamine transporters in human brain. *Life Sci.* **57**, 187–191 (1995).
- Madras, B. K. & Kaufman, M. J. Cocaine accumulates in dopamine-rich regions of primate brain after iv administration: comparison with mazindol distribution. *Synapse* **18**, 261–275 (1994).
- Shyu, B. C., Kiritsy-Roy, J. A., Morrow, T. J. & Casey, K. L. Neurophysiological and behavioral evidence for medial thalamic mediation of cocaine-induced dopaminergic analgesia. *Brain Res.* **572**, 216–223 (1992).
- Groenewegen, H. J. Organization of the afferent connections of the mediodorsal thalamic nucleus in the rat, related to the mediodorsal-prefrontal topography. *Neuroscience* **24**, 379–431 (1988).
- Nauta, W. J. H. The problem of the frontal lobe: a reinterpretation. *J. Psychiatric Res.* **8**, 167–189 (1971).
- McAlonan, G. M., Robbins, T. W. & Everitt, B. J. Effects of medial dorsal thalamic and ventral pallidum lesions on the acquisition of a conditioned place preference: further evidence for the involvement of the ventral striatopallidum system in reward-related processes. *Neuroscience* **52**, 605–620 (1993).
- Kolb, B. Studies on the caudate putamen and the dorsomedial thalamic nucleus: implications for mammalian frontal lobe function. *Physiol. Behav.* **18**, 237–244 (1977).
- Insel, T. R. Towards a neuroanatomy of obsessive-compulsive disorder. *Arch. Gen. Psychiatry* **49**, 739–744 (1992).
- Volkow, N. D. *et al.* Decreased dopamine D2 receptor availability is associated with reduced frontal metabolism in cocaine abusers. *Synapse* **14**, 169–177 (1993).
- Modell, J. G., Mountz, J. M. & Beresford, T. P. Basal ganglia/limbic striatal and thalamocortical involvement in craving and loss of control in alcoholism. *J. Neuropsychiatry* **2**, 123–144 (1990).
- Volkow, N. D. *et al.* Evaluation of the human brain dopamine system with PET. *J. Nucl. Med.* **37**, 1242–1256 (1996).
- Gawin, F. & Kleber, H. D. Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. *Arch. Gen. Psychiatry* **43**, 107–113 (1986).
- Logan, J. *et al.* Graphical analysis of reversible radioligand binding from time-activity measurements applied to [¹¹C]-methyl-(-)-cocaine: PET studies in human subjects. *J. Cereb. Blood Flow Metab.* **10**, 740–747 (1990).

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Vertebrate homologues of *C. elegans* UNC-5 are candidate netrin receptors

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In the developing nervous system, migrating cells and axons are guided to their targets by cues in the extracellular environment. The netrins are a family of phylogenetically conserved guidance cues that can function as diffusible attractants and repellents for different classes of cells and axons^{1–10}. In vertebrates, insects and nematodes, members of the DCC subfamily of the immunoglobulin superfamily have been implicated as receptors that are involved in migration towards netrin sources^{6,11–13,15}. The mechanisms that direct migration away from netrin sources (presumed repulsions) are less well understood. In *Caenorhabditis elegans*,

b

UNC-5 UNC5H1 UNC5H2 UNC-5

Ig

28%

52%

28%

Tsp

ZO-1 Homology

c

1622	AT-ARGIFNSNGGVLS	ETGVSIILPQCAIPEGIEQ	ETVFKVCRDNE	LLPPLDK	ETKGET
496	SNHAYGTNFLLGGRL	MIPTGHSLLIIPDAIPR	GKIYEYLLTHKPF	EDVRLPLA	-GCQT
542	SSSVSGTFFCLGGRL	TIPTGTVSVLLVNGAID	QKFFDYLLRINKT	EST-LPLS	-EGSQT
529	QNI VAAQIDENCA	RLSLSKSGCARLLVPR	LAVEGEKM--LVLA	USDTLTDQPH	KPI--ES

ZO-1	1681	LLSPVLVHGGR	GL-----KFLKRV	ETRDEG
UNC5H1	554	LLSPVVSGGPR	GV-----LLTRPVI	LAHDRC
UNC5H2	600	VLSPSVVCGGPR	GV-----LLCRPVV	ETVPHC
UNC-5	585	ALSPVIVIGNC	DVSMSAHDNILRR	VVVSFRHCA

Figure 1 Structures of UNC5H1 and UNC5H2. **a**, Sequences of the two rat UNC5H proteins compared with that of *C. elegans* UNC-5. Signal peptide sequences in the UNC5H proteins are indicated by a black bar, amino-acid identities by black boxes and similarities by shaded boxes; extents of the two immunoglobulin-like domains (Ig-1 and Ig-2) and the two Tsp type-1 domains (Tsp-1 and Tsp-2) are indicated by arrows; the membrane-spanning region is indicated by asterisks, and the region of homology with ZO-1 is bounded by $\wedge$ symbols. **b**, Predicted topology of UNC-5 and its homologues, indicating the predicted structural domains, ZO-1 homology region, and per cent sequence identity. **c**, Alignment of sequences of UNC5, UNC5H proteins and ZO-1, in their regions of homology; shading as in **a**.

the transmembrane protein UNC-5 (ref. 14) has been implicated in these responses, as loss of *unc-5* function causes migration defects^{16,17} and ectopic expression of *unc-5* in some neurons can redirect their axons away from a netrin source¹⁸. Whether UNC-5 is a netrin receptor or simply an accessory to such a receptor has not, however, been defined. We now report the identification of two vertebrate homologues of UNC-5 which, with UNC-5 and the product of the mouse *rostral cerebellar malformation* gene (*rcm*)¹⁹, define a new subfamily of the immunoglobulin superfamily, and whose messenger RNAs show prominent expression in various classes of differentiating neurons. We provide evidence that these two UNC-5 homologues, as well as the *rcm* gene product, are netrin-binding proteins, supporting the hypothesis that UNC-5 and its relatives are netrin receptors.

Complementary DNAs encoding two rat homologues of UNC-5, UNC5H1 and UNC5H2, were isolated from an embryonic-day (E)18 rat brain cDNA library (see Methods). The predicted proteins show sequence similarity with UNC-5 over the entire length (Fig. 1a), but are more similar to one another (52% identity) than to UNC-5 (28% identity in each case). Like UNC-5 (ref. 14) both have two predicted immunoglobulin-like domains and two predicted thrombospondin type-1 repeats in their extracellular domains, a predicted membrane-spanning region, and a large intracellular domain (Fig. 1b). The UNC5H proteins also each possess a signal sequence which, curiously, is lacking in UNC-5 (ref. 14). The predicted topology of the UNC5H proteins in cell membranes (Fig. 1b) was verified using recombinant versions of the proteins expressed in transfected cells and antibodies directed against the extracellular and intracellular domains (see Methods). The cytoplasmic domains of the two UNC5H proteins do not contain

obvious signalling motifs, but do possess a small region of homology to Zona Occludens-1 (ZO-1) (Fig. 1c), a protein that localizes to adherens junctions and is implicated in junction formation^{20,21}. ZO-1 contains PDZ domains^{20,21}, structures implicated in protein clustering²², but the region of homology with UNC-5 homologues corresponds to a unique sequence at the carboxy terminus of ZO-1 (Fig. 1c). The homology between ZO-1 and *C. elegans* UNC-5 is less pronounced (and was not detected by a computer BLAST search), but is nonetheless apparent when all four sequences are aligned (Fig. 1c). This homology is also evident in the newly identified UNC-5 homologue RCM¹⁹. RCM and UNC5H2 are more similar to one another (66% identity) than either is to UNC5H1 (55% identity between RCM and UNC5H1).

To determine whether UNC5H1 and UNC5H2 are candidates for receptors involved in neuronal migration or axon guidance, we first examined their sites of expression by RNA *in situ* hybridization in rat embryos. *Unc5h1* transcripts are detected at early stages of neural tube development in the ventral spinal cord (Fig. 2a–c). At E11, when motor neurons are beginning to differentiate in that region²³, transcripts are present throughout the ventral spinal cord, excluding the midline floorplate region, but are most intense in the ventricular zone and at the lateral edges (Fig. 2a, and data not shown). At E12, expression is observed in the motor columns and is becoming excluded from the ventricular zone, and also extends more dorsally into a region encompassing the cell bodies of some commissural and association neurons (Fig. 2b). The more dorsal expression appears transient, as expression by E13 is confined to postmitotic cells in the ventral spinal cord, apparently including the motor neurons (Fig. 2c). *Unc5h2* transcripts are not detected at significant levels in the spinal cord until E14, when they are found in the roof plate region (Fig. 2d). They are, however, detected in developing sensory ganglia that flank the spinal cord, at low levels at E12 (data not shown), and at high levels by E14 (Fig. 2d). The expression of these two genes is thus observed in regions where differentiating neurons are undergoing axonogenesis, consistent with a possible role in this process.

These genes are also expressed at higher axial levels of the nervous system, as well as in non-neural structures. At E13, *Unc5h1* is expressed in the basal plate (ventral neural tube) in the hindbrain and midbrain, in the developing hypothalamus and thalamus, and in the pallidum (Fig. 3a). *Unc5h2* expression at this stage is detected in the nervous system in the dorsal aspect of the developing optic cup and in restricted regions of the midbrain and caudal diencephalon (Fig. 3b, and data not shown). By E16, *Unc5h1* mRNA is strong in the entorhinal cortex and weaker throughout the cortex (data not shown). Small amounts of *Unc5h2* are also detected at this stage in the cortex, and in large amounts in hypertrophic chondrocytes (data not shown). Expression of the two homologues persists postnatally, with, at postnatal-day 10 (P10), continued expression of both at low levels throughout the cortex, expression of both in distinct patterns in the septal area, and high level expression of *Unc5h1* in the developing hippocampus and entorhinal cortex (Fig. 3c, d). In addition, a prominent site of postnatal expression of both genes is in the cerebellum (Fig. 3e–f). Both are expressed in the inner granule cell layer (Fig. 3e, f), and *Unc5h2* is expressed in the inner aspect of the external germinal layer, where granule cell precursors differentiate before migrating to their final destination in the inner granule cell layer, as well as by some cells in the molecular layer, which might represent migrating granule cells (Fig. 3f)^{24,25}.

Thus, the expression patterns of the two *Unc5h* genes are suggestive of potential roles in cell or axon migration. It is particularly striking that *Unc5h2* expression overlaps with *rcm* in granule cells that are about to undergo a migration that is defective in *rcm*-mutant mice¹⁹. The co-expression of these related proteins might account for the fact that only a subset of *rcm*-expressing cerebellar cells are affected in *rcm*-null mutants¹⁹.

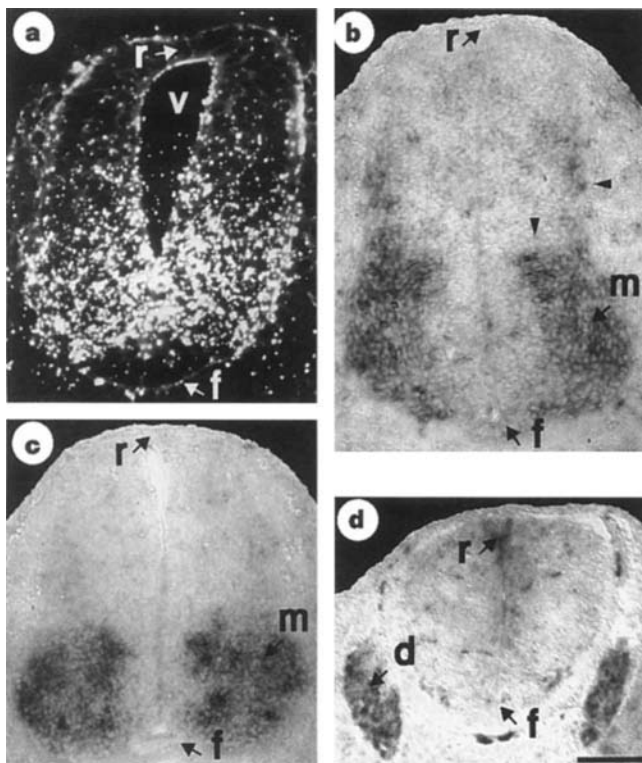


Figure 2 a–c, Expression of *Unc5h1* mRNA in transverse sections through the developing rat spinal cord at E11 (a), E12 (b) and E13 (c). Note that at E12, expression is observed in the motor column (m), but also in more medially positioned cells in the ventral spinal cord (vertical arrowhead) as well as more dorsally (horizontal arrowhead). d, expression of *Unc5h2* in developing sensory ganglia and roof plate of the spinal cord in an E14 rat embryo. Abbreviations: d, dorsal root ganglia; f, floor plate; m, motor column; r, roof plate; v, ventricular zone. Scale bar shown in d is 30, 100, 130 and 320 μ m in a, b, c and d, respectively.

To test whether UNC-5 homologues might be involved in mediating responses to netrins, we tested whether netrin-1 can bind cells expressing these proteins. Transfected human embryonic kidney 293 cells expressing UNC5H1, UNC5H2 or RCM showed binding of netrin-1 protein that was significantly above background (Fig. 4a–d, and data not shown), as is also observed for transfected cells expressing the netrin receptors DCC and neogenin, but not for transfected cells expressing TAG-1 or L1, two other members of the immunoglobulin superfamily¹³. In these experiments, binding was performed in the presence of soluble heparin, which eliminates nonspecific binding of netrin-1 to the cells¹³ but does not prevent binding to the UNC5 homologues. Binding was similarly specific when cells were incubated with netrin-1 without added heparin and subsequently washed with only heparin-containing medium (data not shown), indicating that heparin is not required for the binding interaction. To verify this, in the case of UNC5H2, we showed that exogenously added heparin is not required for the interaction by generating a soluble protein comprising the extracellular domain of UNC5H2 fused to the constant region (Fc) of a human immunoglobulin molecule. This UNC5H2–Fc fusion protein bound transfected 293 cells expressing netrin-1 (some of which remains

associated with the surface of these cells^{3,10}) in the absence of added heparin (Fig. 4e) but did not bind to non-transfected cells (Fig. 4f) or to cells expressing UNC5H2 itself, DCC or neogenin (data not shown). In addition, the UNC5H2–Fc fusion did not bind transfected cells expressing F-spondin, an adhesive extracellular matrix protein made by floorplate cells²⁶, or semaphorin III, a chemorepellent for sensory axons at the stages that *Unc5h2* is expressed in sensory ganglia²⁷ (data not shown). Both of these proteins, like netrin-1, are secreted, but partition between cell surfaces and the soluble fraction^{26,28}. Thus, the interaction between netrin-1 and UNC5H2 appears to be specific and does not require heparin or reflect a generalized interaction with proteins that associate nonspecifically with cell surfaces.

The affinity of UNC-5 homologues for netrin-1 was estimated in equilibrium binding experiments using netrin(VI·V)–Fc, a fusion of the amino-terminal two-thirds of netrin-1 to the constant portion of human IgG¹³. This netrin-1 derivative is bioactive but, unlike netrin-1, does not aggregate at high concentrations, and it binds DCC with a dissociation constant (K_d) comparable to that of full-length netrin-1 (ref. 13). Specific binding of netrin (VI·V)–Fc to each of the three UNC5 homologues showed saturation and the

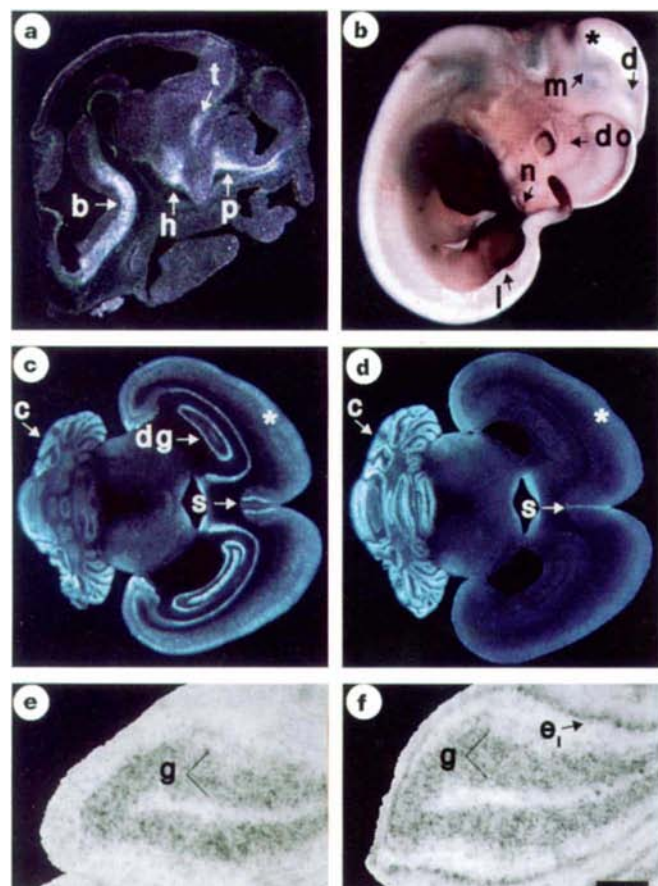


Figure 3 Other sites of expression of *Unc5h1* (a, c, e) and *Unc5h2* (b, d, f). a, b, Expression at E13, visualized in a parasagittal section through the head (a) or in an embryo whole-mount (b). Asterisk in b indicates expression at the midbrain hindbrain junction. c, d, Semi-adjacent horizontal sections of P10 brain. Asterisks indicate expression in cerebral cortex. e, f, Semi-adjacent parasagittal sections through folia in the cerebellum. Abbreviations: l, limb; b, basal plate; c, cerebellum; d, caudal diencephalon; do, dorsal aspect of the optic cup; dg, dentate gyrus; ei, inner aspect of the external germinal layer; g, inner granule cell layer; h, hypothalamus; m, ventral aspects of midbrain; n, nasal pits; p, pallidum; s, septal region. Scale bar shown in f is 500 μ m in a, 6.6 mm in b, 160 μ m in c and d, and 200 μ m in e and f.

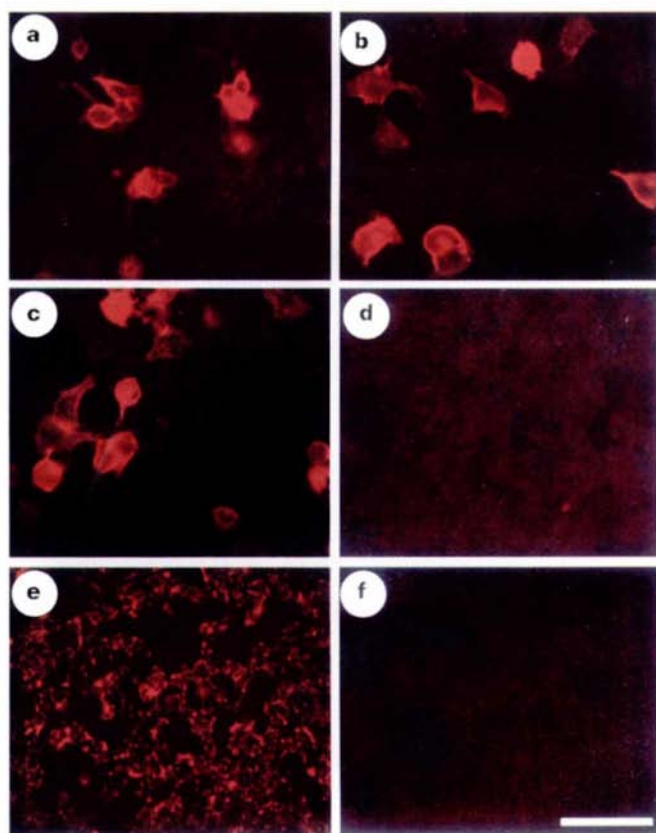


Figure 4 Interactions of netrin-1 and UNC5H proteins, as demonstrated in experiments in which purified netrin-1 (a–d) is incubated with cells expressing candidate receptors or in which a soluble UNC5H2-ectodomain–Fc fusion protein is incubated with cells expressing netrin-1 (e–f). a–f, Fluorescence photomicrographs. a–c, Binding of netrin-1, detected with antibody 9E10 against a C-terminal myc-epitope on the protein, to 293T cells expressing UNC5H1 (a), UNC5H2 (b) or RCM (c). d, In control experiments, netrin-1 did not show significant binding to 293T cells expressing the Ig superfamily member L1. Note that in these transient transfections, only ~10% of cells express the constructs. e, f, An UNC5H2-ectodomain–Fc fusion protein binds 293 cells stably expressing netrin-1 (e) but not the parental 293 cell line (f). Note that all cells express netrin-1 on their surface in this case. Scale bar in a–d is 66 μ m, and 200 μ m in e and f.

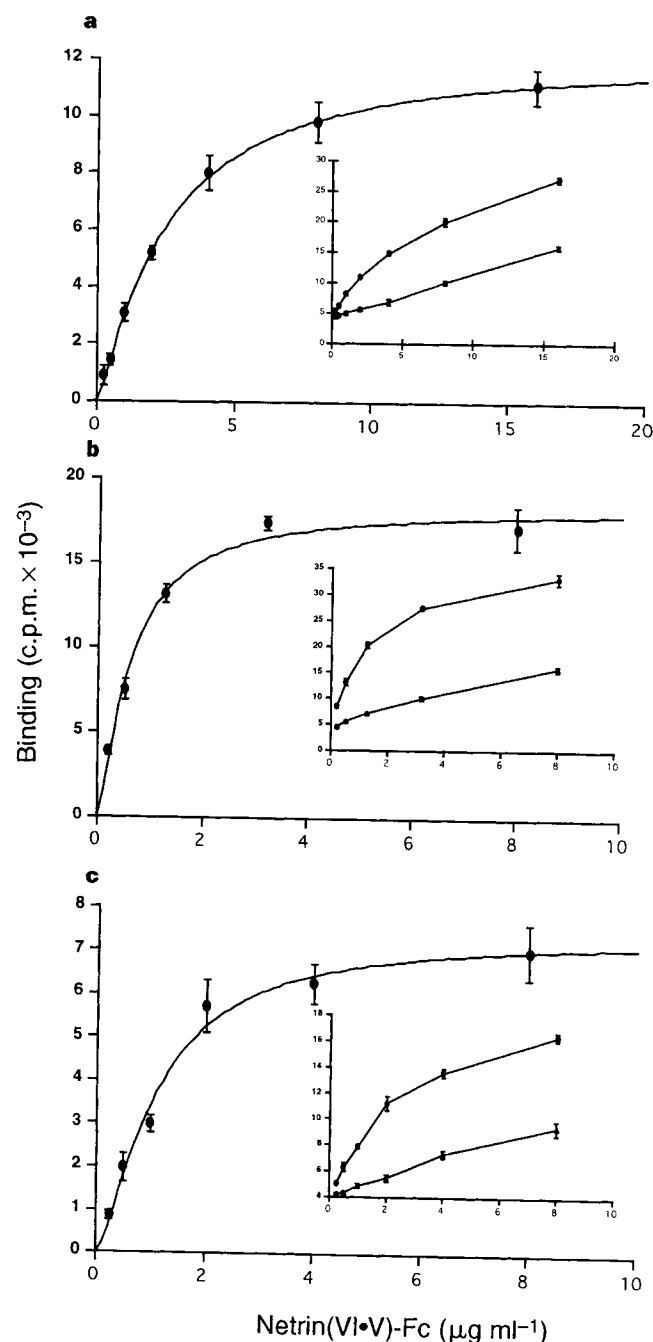


Figure 5 a–c. Equilibrium binding of the netrin (VI-V)-Fc fusion protein to UNC5H1 (a), UNC5H2 (b) and RCM (c). Binding of netrin (VI-V)-Fc was determined by measuring the radioactivity associated with the cells after subsequent incubation with radiolabelled anti-human IgG antibody. Insets show raw data (circles, total binding to receptor-expressing cells; triangles, total binding to mock-transfected cells). Specific binding curves were fitted using the Hill equation (larger-scale graph in each panel). K_d values for the interaction of netrin (VI-V)-Fc with UNC5H1, UNC5H2 and RCM were $3.04 \pm 0.13 \mu\text{g ml}^{-1}$, $0.54 \pm 0.17 \mu\text{g ml}^{-1}$ and $1.14 \pm 0.29 \mu\text{g ml}^{-1}$, respectively ($1 \mu\text{g ml}^{-1}$ corresponds to 6.25 nM). Bars indicate s.e.m. for triplicates. Results are from one representative of two (UNC5H2) or three (UNC5H1, RCM) experiments.

binding curves were fitted to the Hill equation (Fig. 5a–c), yielding K_d values of $19 \pm 0.8 \text{ nM}$, $3.4 \pm 1.0 \text{ nM}$ and $6.9 \pm 1.8 \text{ nM}$ for UNC5H1, UNC5H2 and RCM respectively. These values are comparable to the K_d for the DCC/netrin (VI-V)-Fc interaction ($\sim 5 \text{ nM}$) and are consistent with the effective dose for the axon-outgrowth-promoting effects of netrin-1 (refs 2, 13).

The phenotype of *rcm* mutant mice implicates RCM in cell migration¹⁹, but establishing the involvement of UNC5H1 and UNC5H2 in cell migration and axon guidance will require perturbing their functions *in vivo*. In the meantime, however, our results are at least consistent with such an involvement, as these homologues are expressed by some populations of cells that are undergoing migrations or extending axons. For example, *Unc5h1* is expressed by spinal motor neurons, whose axons are repelled *in vitro* by floor-plate cells²⁹ and whose outgrowth *in vitro* can be suppressed by netrin-1 (ref. 30). It is also expressed in the region of trochlear motor neurons, which can be repelled by netrin-1 (refs 4, 10, 30).

Our evidence that vertebrate UNC5H proteins bind netrin-1 provides direct support for the idea that members of this new subfamily of the immunoglobulin superfamily are netrin receptors. This idea was first proposed for *C. elegans* UNC-5, based on the findings that *unc-5* is needed cell-autonomously for dorsal migrations that require the function of the netrin UNC-6 (refs 14, 16), and that ectopic expression of *unc-5* in neurons that normally project longitudinally or ventrally can steer their axons dorsally¹⁸. Although consistent with the possibility that UNC-5 is an UNC-6 receptor, these results are also consistent with a role for UNC-5 in modifying the function of a different UNC-6 receptor. A modifier function was made more plausible by evidence that the DCC homologue UNC-40, a putative UNC-6 receptor involved in ventral migrations¹¹, is expressed by axons that project dorsally and is required for those projections^{11,16,17}, suggesting that UNC-5 might function by switching an attractive netrin receptor (UNC-40) into a repulsive netrin receptor. Although such a switching role is possible, our results suggest that UNC-5 might itself also function directly as a netrin receptor. A model in which UNC-40 and UNC-5 can form a receptor complex but UNC-5 can also function alone in transducing the UNC-6 netrin signal would explain why loss of *unc-40* function results in a much less severe phenotype for dorsal migrations than do either loss of *unc-5* or loss of *unc-6* function^{16,17}. In this context, it is notable that *Unc5h1* and *Unc5h2* are expressed in regions of the vertebrate nervous system where the *unc-40* homologues DCC and *neogenin* are expressed (for example, in dorsal and ventral spinal cord, dorsal retina, dorsal root ganglia and cerebellum (refs 13, 31, and data not shown). Thus, a model in which UNC-5 and UNC-40 form a receptor complex might also be applicable to vertebrates.

There is a remarkable phylogenetic conservation in function of netrin proteins in guiding axons towards a source of netrin at the midline of the nervous systems of nematodes, flies and vertebrates^{1,7,8,9}, as well as a conserved role for members of the DCC subfamily of the immunoglobulin superfamily in mediating the axonal responses that underlie those guidance events^{11–13}. Identification of vertebrate homologues of UNC-5, and the evidence that they are netrin-binding proteins, raises the possibility that the signalling mechanisms through which netrins elicit repulsive responses are also conserved. □

Methods

Isolation of rat UNC-5 homologues and *in situ* hybridization. A search of the human expressed sequence tag (EST) databases revealed a small sequence (Genbank accession number R11880) with distant similarity to the C-terminal portion of UNC-5. The corresponding cDNA fragment, amplified by polymerase chain reaction (PCR) from an embryonic human brain cDNA library (Stratagene) was used to screen the library, resulting in the isolation of a 3.8 kb cDNA clone comprising all but the first 440 nucleotides of the coding region of the human homologue of *Unc5h1*. Non-overlapping probes from this cDNA were used to screen an E18 rat brain library (gift from S. Nakanishi),

leading to the isolation of seven partial and one full-length *Unc5h1* cDNA and one full-length *Unc5h2* cDNA. Additional screens of E13 rat dorsal and ventral spinal cord libraries resulted in isolation of a second full-length *Unc5h2* cDNA as well as a nearly full-length *Unc5h1* cDNA. Sequencing was done on a Licor (L4000) automated sequencer as well as by ³³P cycle sequencing. Genbank accession numbers are U87305 and U87306 for rat *Unc5h1* and *Unc5h2*, respectively. RNA *in situ* hybridization was performed as described¹³.

Antibodies, expression constructs and immunohistochemistry. Rabbit polyclonal antisera were raised against a peptide corresponding to a sequence (YLRKNFEQEPLAKE) in the extracellular domain of UNC5H1 that is almost completely conserved in UNC5H2 (one amino-acid substitution) and to peptides corresponding to unique sequences in the cytoplasmic domains of UNC5H1 (GEPSPDSWSRLRKQ) and UNC5H2 (EARQQDDGDLNSLASA). Antisera were affinity-purified on their respective peptides (Quality Controlled Biochemicals). cDNAs for the various constructs were subcloned into the expression vectors pMT21 (Genetics Institute) and pCEP4 (Invitrogen) (for UNC5H1 and UNC5H2) or pRC/CMV (Invitrogen) (for RCM), and transiently transfected into 293 cells using lipofectamine. The antiserum to the extracellular peptide can detect UNC5H1 and UNC5H2 proteins expressed in transfected cells without cell permeabilization, whereas the antisera directed against the cytoplasmic domain peptides detected their respective proteins after cell permeabilization (data not shown). Netrin-1 protein was produced, purified, used and visualized in binding assays as described¹³, except that a monoclonal antibody (9E10)³² directed against a C-terminal Myc-epitope tag was used to detect recombinant netrin-1. Binding with netrin (VI-V)-Fc on 293T cells transiently transfected with UNC5H1, UNC5H2 or RCM was quantified essentially as described¹³, except that after incubation with ligand, cells were washed once rapidly with 600 µl PBS and then fixed with 100% methanol followed by 4% paraformaldehyde. Cells were then washed once in PBS before proceeding with the secondary antibody incubation. The full-length UNC5H1 protein appears to be toxic for transfected 293 cells (data not shown), so binding experiments used a truncated UNC5H1 protein lacking a portion of the cytoplasmic domain (truncation at amino acid 494), which was not toxic. A 293-EBNA cell line stably expressing the UNC5H2-Fc fusion was derived and maintained as described^{10,13}. The fusion protein was purified from serum-free medium conditioned for 7 d by affinity chromatography on protein A-agarose. The 293 cell line expressing netrin-1 has been described¹⁰. Binding of the UNC5H2-Fc fusion to this line was visualized using a Cy3-conjugated secondary antibody (Jackson Immunoresearch) directed against human Fc.

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- Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G. & Hedgecock, E. M. UNC-6, a laminin related protein, guides cells and pioneer axon migrations in *C. elegans*. *Neuron* **9**, 873–881 (1992).
- Serafini, T. *et al.* The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78**, 409–424 (1994).
- Kennedy, T. E., Serafini, T., de la Torre, J. R. & Tessier-Lavigne, M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425–435 (1994).
- Colamarino, S. A. & Tessier-Lavigne, M. The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell* **81**, 621–629 (1995).
- Shirasaki, R., Tamada, A., Katsumata, R. & Murakami, F. Guidance of cerebellar axons in the rat embryo: directed growth toward the floor plate and subsequent elongation along the longitudinal axis. *Neuron* **14**, 961–972 (1995).
- Wadsworth, W. G., Bhatt, H. & Hedgecock, E. M. Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in *C. elegans*. *Neuron* **16**, 35–46 (1996).
- Mitchell, K. J. *et al.* Genetic analysis of *Netrin* genes in *Drosophila*. Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* **17**, 203–215 (1996).
- Harris, R., Sabatelli, L. M. & Seeger, M. A. Guidance cues at the *Drosophila* CNS midline: identification and characterization of two *Drosophila* Netrin/UNC-6 homologs. *Neuron* **17**, 217–228 (1996).
- Serafini, T. *et al.* Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001–1014 (1996).
- Shirasaki, R., Mirzayan, C., Tessier-Lavigne, M. & Murakami, F. Guidance of circumferentially growing axons by netrin-dependent and -independent floor plate chemotropism in the vertebrate brain. *Neuron* **17**, 1079–1088 (1996).
- Chan, S. S.-Y. *et al.* UNC-40, a *C. elegans* homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell* **87**, 187–195 (1996).
- Kolodziej, P. A. *et al.* *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell* **87**, 197–204 (1996).
- Keino-Masu, K. *et al.* Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* **87**, 175–185 (1996).
- Leung-Hagestijn, C. *et al.* UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in *C. elegans*. *Cell* **71**, 289–299 (1992).
- Fazeli, A. *et al.* Phenotype of mice lacking functional Deleted in Colorectal Cancer (DCC) gene. *Nature* **386**, 796–804 (1997).
- Hedgecock, E. M., Culotti, J. G. & Hall, D. H. The *unc-5*, *unc-6* and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* **2**, 61–85 (1990).
- McIntire, S. L., Garriga, G., White, J., Jacobson, D. & Horvitz, H. R. Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron* **8**, 307–322 (1992).

- Hamelin, M., Zhou, Y., Su, M. W., Scott, I. M. & Culotti, J. G. Expression of the UNC-5 guidance receptor in the touch neurons of *C. elegans* steers their axons dorsally. *Nature* **364**, 327–330 (1993).
- Ackerman, S. L. *et al.* The mouse rostral cerebellar malformation gene encodes an UNC-5 like protein. *Nature* **386**, 838–842 (1997).
- Willott, E. *et al.* The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions. *Proc. Natl Acad. Sci. USA* **90**, 7834–7838 (1993).
- Itoh, M. *et al.* The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* **121**, 491–502 (1993).
- Sheng, M. PDZs and receptor/channel clustering: rounding up the latest suspects. *Neuron* **17**, 575–578 (1996).
- Altman, J. & Bayer, S. A. The development of the rat spinal cord. *Adv. Anat. Embryol. Cell Biol.* **85**, 1–166 (1984).
- Ramón y Cajal, S. *Histologie du Système Nerveux de l'Homme et des Vertébrés* Vol. 2 (Maloine, Paris, 1911).
- Rakic, P. Neuron–glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electron microscopic study in *Macacus rhesus*. *J. Comp. Neurol.* **141**, 283–312 (1971).
- Klar, A., Baldassare, M. & Jessell, T. M. F-spondin: a gene expressed at high levels in the floor plate encodes a secreted protein that promotes neural cell adhesion and neurite extension. *Cell* **69**, 95–110 (1992).
- Messersmith, E. K. *et al.* Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron* **14**, 949–959 (1995).
- Luo, Y., Raible, D. & Raper, J. A. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**, 217–227 (1993).
- Guthrie, S. & Pini, A. Chemorepulsion of developing motor axons by the floor plate. *Neuron* **14**, 1117–1130 (1995).
- Varela-Echavarría, A., Tucker, A., Puschel, A. & Guthrie, S. Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. *Neuron* **18**, 193–207 (1997).
- Livesey, F. J. & Hunt, S. P. Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests role in retinal, striatal, nigral and cerebellar development. *Mol. Cell. Neurosci.* (in the press). **UPDATE?**
- Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610–3616 (1985).

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The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein

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Migration of neurons from proliferative zones to their functional sites is fundamental to the normal development of the central nervous system^{1,2}. Mice homozygous for the spontaneous rostral cerebellar malformation mutation (*rcm*^s) or a newly identified transgenic insertion allele (*rcm*^{tg}) exhibit cerebellar and midbrain defects, apparently as a result of abnormal neuronal migration. Laminar structure abnormalities in lateral regions of the rostral cerebellar cortex have been described in homozygous *rcm*^s mice³. We now demonstrate that the cerebellum of both *rcm*^s and *rcm*^{tg} homozygotes is smaller and has fewer folia than in the wild-type, ectopic cerebellar cells are present in midbrain regions by three days after birth, and there are abnormalities in postnatal cerebellar neuronal migration. We have cloned the *rcm* complementary DNA, which encodes a transmembrane receptor of the immunoglobulin superfamily. The sequence of the *rcm* protein (Rcm) is highly similar to that of UNC-5, a *Caenorhabditis elegans* protein that is essential for dorsal guidance of pioneer axons and for the movement of cells away from the netrin ligand, which is encoded by the *unc-6* gene^{4–7}. As Rcm is a member of a newly

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described family of vertebrate homologues of UNC-5 which are netrin-binding proteins, our results indicate that UNC-5-like proteins may have a conserved function in mediating netrin-guided migration⁸.

Ataxia was observed in some of the offspring from heterozygous matings of one (line 1.23) of three lines of mice transgenic for a mitochondrial uncoupling protein (*Ucp*) minigene. Histological analysis of these homozygous transgenic mice revealed cerebellar abnormalities. Complementation tests in which mice heterozygous for the transgenic lineage 1.23 were mated to heterozygous *rcm*^s mice, produced several offspring exhibiting the abnormal gait characteristic of both homozygous 1.23 transgenic mice and homozygous *rcm*^s mice. On the basis of these results, we assumed that the *Ucp* minigene disrupted the *rcm* locus in this lineage.

A dramatic reduction in cerebellar size and in the number of folia was observed in midline sagittal sections of cerebella from homozygous *rcm*^{tg} and *rcm*^s adults (Fig. 1). This size reduction was more pronounced in *rcm*^{tg} than in *rcm*^s homozygotes. Vermal fissure development in *rcm*^{tg} and *rcm*^s homozygotes resulted in the formation of only five or six lobes, respectively, compared with the eight major lobes seen in the control wild-type mice, *rcm*^{tg}/+ and +/+ (B6C3). Fissure formation was also abnormal in the lateral cerebellar hemispheres of both *rcm*^s/*rcm*^s and *rcm*^{tg}/*rcm*^{tg} mice, a disruption that was more severe in the *rcm*^{tg} homozygote (Fig. 1). The effects of both the *rcm*^{tg} and *rcm*^s mutations on fissure development were evident at birth (P0) and fissure development continued to be delayed during the postnatal period. The cerebella of mutant mice were smaller at birth, suggesting that the *rcm* gene may play a role in cell proliferation during embryogenesis (data not shown).

The most striking defect in the brain of *rcm* homozygotes was found in lateral regions where the inferior colliculus adjoins the cerebellum. An abnormal band of granule cells extending from the cerebellum and continuing into the inferior colliculus was observed in sagittal sections lateral to midline. Analysis of the more lateral sections revealed partial fusion of the inferior colliculus and the rostral cerebellum and a number of ectopic granule and calbindin-positive Purkinje cells in the inferior colliculus and tegmentum of the midbrain (Fig. 1, and data not shown). These ectopic cells were not found in the adjoining superior colliculus or thalamus, suggesting that incursion of cerebellar cells into these regions is blocked during brain development. To determine when during development these ectopic granule and Purkinje cells arise, we did an *in situ* analysis of serial sections from *rcm*^s/*rcm*^s embryos at embryonic days 13.5, 15.5 and 17.5 and at postnatal days P0, P3 and P7, using an antisense probe for *Math1*, a basic helix-loop-helix protein expressed in external granular cells^{9,10}. Ectopic granule cells were first detected at P3, the time when these cells normally begin their postnatal migration, and had increased in number by P7 (Fig. 2a). Ectopic Purkinje cells were not detected at P0 but were found at P3 (Fig. 2b). Both ectopic granule and Purkinje cells expressed the *rcm* transcript (data not shown), supporting a cell-autonomous role for the *rcm* protein.

Defects in the laminar structure of *rcm*^{tg}/*rcm*^{tg} and *rcm*^s/*rcm*^s cerebella were observed by P7. Abnormal external granule cell migration, characterized by the appearance of cohorts rather than individual cells migrating across the molecular layer, was clearly evident in some lateral areas of the cerebellum (Fig. 2c). These aggregates of cells that extend into the inner granule layer were often associated with gaps in the Purkinje cell layer. The movement of ectopic Purkinje and granule cells into the midbrain by P3, together with the presence of aberrant cohorts of granule cells postnatally and the abnormal laminar structure in the adult cerebellum, suggest a role for the *rcm* gene in neuronal migration.

To isolate the *rcm* gene, a genomic DNA fragment flanking the transgene insertion site was identified (Fig. 3a). Restriction-fragment length polymorphisms were detected by analysis of DNA from

rcm^{tg} homozygotes and wild-type mice using the flanking fragment as a probe. Analysis of DNA from an interspecific backcross mapping panel confirmed that this fragment was derived from the region of chromosome 3 known to contain the *rcm* gene¹¹. Two P1 clones containing this flanking sequence were identified and genomic fragments that overlapped with the 8-kilobase (kb) *Xba*I fragment were isolated and used as probes on Southern blots containing wild-type and *rcm*^{tg}/*rcm*^{tg} genomic DNA. Both this analysis and polymerase chain reaction (PCR) studies using primers corresponding to these fragments indicated that ~8.5 kb of DNA adjacent to the transgene insertion site was deleted (Fig. 3a, dashed line).

Northern blots of adult brain RNA were probed with genomic DNA fragments corresponding to sequences within the deletion in

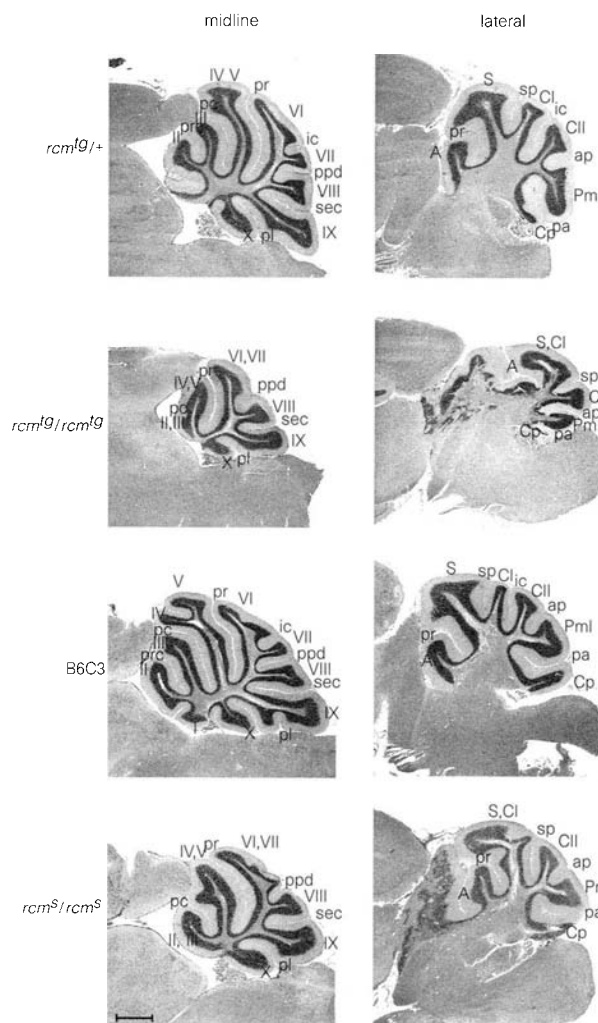


Figure 1 Defects in the *rcm* gene result in abnormalities in foliation and laminar structure of the adult cerebellum. Haematoxylin- and -eosin-stained sagittal sections from midline and lateral regions of wild-type (*rcm*^{tg}/+ and B6C3 Fe a/a (B6C3)) or mutant (*rcm*^{tg}/*rcm*^{tg} and *rcm*^s/*rcm*^s) cerebella are shown. The lateral ventricles and hippocampus were used as landmarks for the lateral sections. Anterior is to the left and dorsal to the top in each photograph. Roman numerals I–X indicate vermal lobes. Fissures are abbreviated as follows: prc, precentral; pc, posterolateral; sp, superior posterior; ap, ansoparamedial; and pa, parafoccularis. Hemispheric lobes are as follows: A, anterior; S, simplex; Cl, crus I; CII, crus II; Pml, paramedial. In midline sections, note the absence of the precentral fissure in the anterior region and the intercrural fissure in the medial posterior region (in midline and lateral sections) in both *rcm*^{tg} and *rcm*^s homozygotes and the poor development of the preculminate fissure in *rcm*^{tg} homozygotes. Scale, 670 μm.

order to identify candidate exons. Both the 1.7-kb *Xba*I fragment (Fig. 3a, hatched box) and an adjacent 5.5-kb *Xba*I fragment (not shown) detected a transcript of ~9.3 kb in wild-type adult brain. DNA sequence analysis of the 1.7-kb *Xba*I fragment using the GRAIL 1A gene analysis program indicated the presence of a 110-base-pair (bp) putative exon. cDNA clones encompassing 9.3 kb of sequence were isolated by 5' and 3' RACE (rapid amplification of cloned ends) using adult brain cDNA. A corresponding transcript was not detected by northern blot analysis of adult cerebellar messenger RNA isolated from homozygous *rcm*^{ts} animals, whereas

a similar amount of transcript was detected in *rcm*^{ts/}*rcm*^{ts} and wild-type adults (Fig. 3b).

To test the *rcm*^{ts} transcript for mutations, PCR analysis with reverse transcription (RT-PCR) was done on *rcm*^{ts/}*rcm*^{ts} and wild-type cerebellar cDNA with primers designed from the putative open reading frame. Primers corresponding to a region of the candidate gene encoding the C terminus of the protein amplified a band from *rcm*^{ts} that was ~160 bp larger than that from wild-type cDNA (data not shown). Sequence analysis revealed a tandem duplication of an exon encoding amino acids 763–818 that did not disrupt the

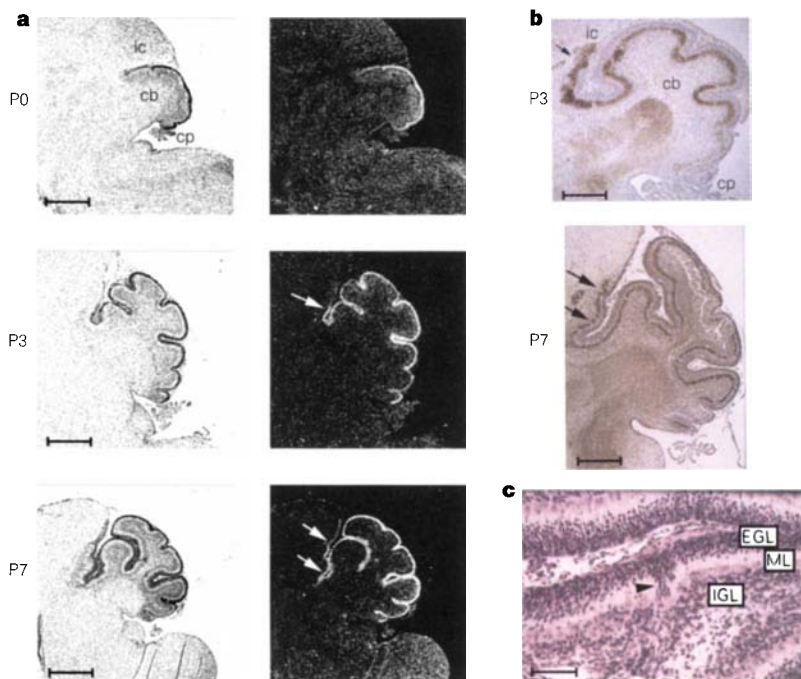


Figure 2 Midbrain and neuronal migration defects in *rcm*^{9/}*rcm*^{9/} mice. Anterior is to the left and dorsal to the top. **a**, *In situ* analysis of parasagittal sections through the inferior colliculus (ic) and cerebellum (cb) using *Math1* as probe. Corresponding bright-field and dark-field images are shown. Arrows denote granule cells in midbrain regions of *rcm*^{9/}*rcm*^{9/} animals. Such cells were not detected in wild-type controls. Scale bars, 5.5 mm in P0 and P3, and 8.0 mm in P7. **b**, Immunohistochemical analysis of Purkinje cells in parasagittal sections of postnatal *rcm*^{9/}*rcm*^{9/} brains after reaction with calbindin antiserum. Sections were counterstained with haematoxylin to show granule cells. Scale bars, 3.0 mm (P3) and 4.8 mm (P7). **c**, Parasagittal section through a P7 cerebellum. Note the presence of an abnormal cohort of granule cells (large arrowheads) and normal, individual granule cells (small arrowheads) in the molecular layer, EGL, external granule layer; ML, molecular layer; IGL, internal granule layer. Scale bar, 1.6 mm.

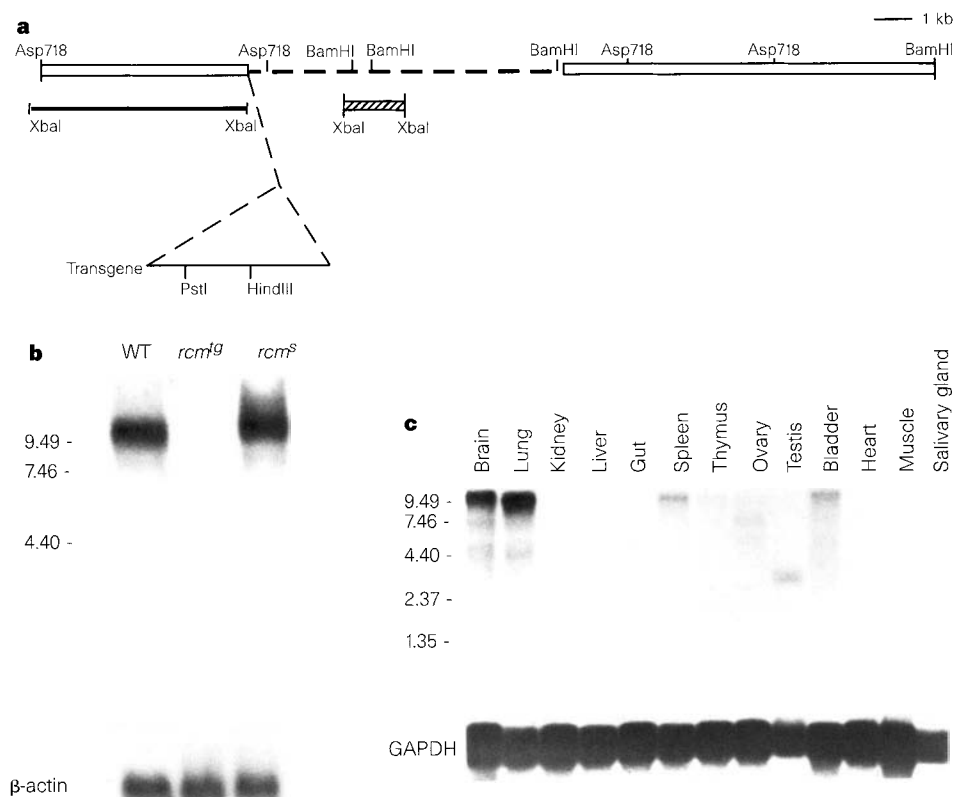


Figure 3 Molecular characterization of the *rcm*^{9/} mutation and the expression pattern of the *rcm* transcript in adult tissues and developing cerebellum. **a**, Map of the genomic DNA surrounding the *Ucp1* transgene insertion. White bars represent genomic DNA, dashed line indicates the 8.5-kb deletion adjacent to the transgene insertion. Solid line represents the original 8-kb *Xba*I fragment isolated from the *rcm*^{9/} genomic library; the hatched box indicates the 1.7-kb *Xba*I fragment containing the exon identified by GRAIL. **b**, Northern blot analysis of 10 µg poly(A)⁺ RNA from the cerebella of wild-type (WT), *rcm*^{9/}*rcm*^{9/}, and *rcm*^{9s/}*rcm*^{9s/} mice. The blot was probed with a 0.7-kb *rcm* partial cDNA fragment and reprobed with β-actin as a control. **c**, Northern blot of poly(A)⁺ RNA (10 µg) extracted from normal adult mouse tissues hybridized to a ³²P-labelled 582-bp *rcm* cDNA fragment. The blot was exposed to X-ray film at -70 °C for 2.5 days. Lower panel, same blot rehybridized to a GAPDH probe and exposed for 5 h. RNA size markers (Gibco BRL) are shown on the left.

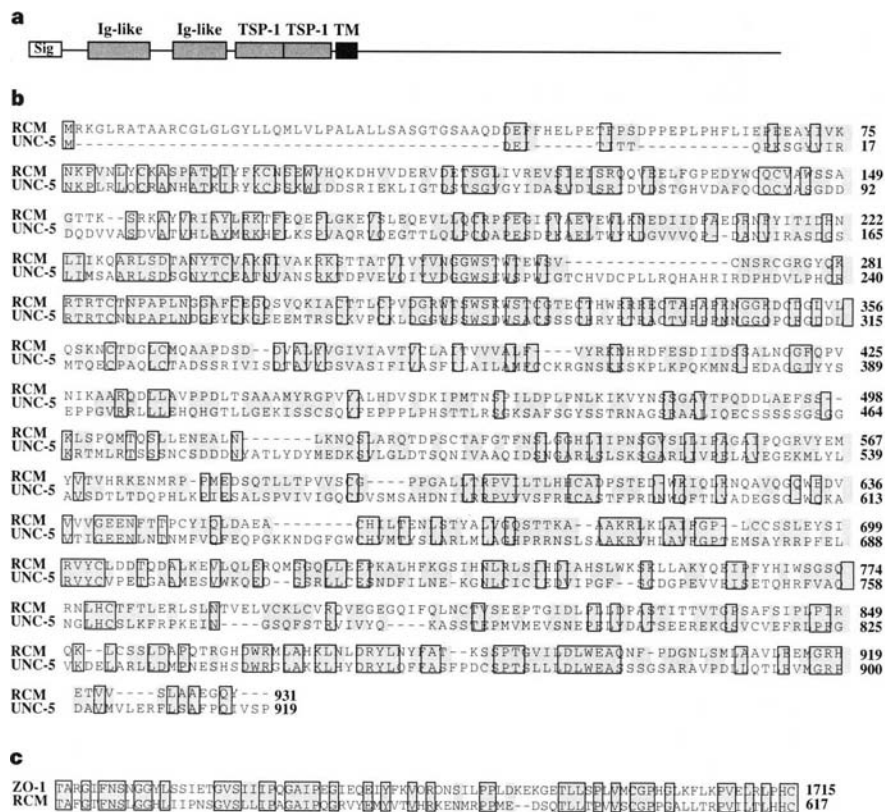


Figure 4 RCM protein structure. **a**, Domain structure of the RCM protein. An N-protein signal sequence (Sig) with a consensus cleavage site at residue 40 was detected by hydrophobicity and signal analysis^{21,22}. A hydrophobic region of 24 amino acids (residues 377–400), predicted to be the single membrane-spanning region based on ALOM analysis, is flanked by charged residues on both the N- and the C-terminal sides, indicating that the RCM protein adopts a type Ia ($N_{\text{exo}}C_{\text{cyto}}$) orientation^{23,24}. TSP-1, thrombospondin type-1; TM, transmembrane domain. **b**, RCM and UNC-5 amino-acid sequence homology. Identical (boxed and shaded) and similar (shaded only) amino acids between the mouse RCM and *C. elegans* UNC-5 proteins. Alignment was performed using the ALIGN program (FASTA). The lack of homology in the most N-terminal region of the RCM protein probably reflects the putative signal sequence in the RCM protein that is not found in UNC-5. **c**, RCM and ZO-1 amino-acid sequence homology. Identical (shaded and boxed) and similar (shaded only) amino acids in the C-terminal region of the mouse RCM and the mouse ZO-1 proteins. The region is also conserved in the human ZO-1 protein.

reading frame but resulted in a putative Rcm protein containing an additional 55 amino acids. Southern blot analysis of wild-type and homozygous *rcm*^s DNA using this exon as a probe confirmed the presence of this duplication in the genome of *rcm*^s/*rcm*^s mice (data not shown).

Northern blot analysis of the *rcm* transcript in adult mouse tissues indicated that expression was highest in brain and lung (Fig. 3c). Less *rcm* RNA was detected in testis, ovary, thymus, spleen and bladder; upon longer exposure (>7 days), transcripts were also detected in kidney, intestine and salivary gland. In addition to the predominant 9.3-kb band seen in most tissues, two additional transcripts of 6.0 kb and 4.4 kb were present from ovary; testis expresses a single band of 3.4 kb.

Sequence analysis of *rcm* cDNA clones detected an open reading frame encoding a 931-amino-acid transmembrane protein with a predicted relative molecular mass of 103K. BLASTP analysis detected two immunoglobulin (Ig)-like domains (residues 76–151 and 181–246) and two thrombospondin (TSP) type-1 motifs (residues 259–315 and 316–369) within the 377-amino-acid extracellular region (Fig. 4a). This unique combination of immunoglobulin and TSP-1 domains in the extracellular region is very similar to the *C. elegans* UNC-5 transmembrane protein involved in cell migration⁹. Amino-acid sequence comparison of the Rcm and UNC-5 transmembrane protein also revealed a high homology (29.5% identity and 72.8% similarity between residues 66–931) (Fig. 4b). This sequence and structure homology between Rcm and UNC-5 strongly supports our idea that Rcm is involved in cerebellar neuronal migration. Although the intracellular region of Rcm appears to have no discernible domain structure, the amino-acid sequence between residues 531–612 is 48% identical (65% similarity) to the mouse and human tight-junction protein ZO-1 (Fig. 4c). ZO-1 contains both SH3 and guanylate kinase-like domains, and may be involved in signal transduction at specialized cell–cell junctions¹².

The *rcm* transcript is temporally and spatially regulated during postnatal granule cell migration. *In situ* hybridization analysis of P0

brain detected high levels of *rcm* mRNA in the cerebellum (Fig. 5a). Cerebellar *rcm* expression appeared to peak at P7 (Fig. 5d) and was significantly downregulated by P21 (Fig. 5f), by which time granule-cell migration is complete. In the P0 cerebellum, *rcm* expression was detected in granule-cell precursors in the external granule layer as well as in Purkinje cells. At P7, *rcm* expression was detectable in cells of both the superficial proliferating zone of the external granule layer and in those of the premigratory zone. Expression was also seen in migrating granule cells within the molecular layer, post-migratory cells in the internal granule layer, and in Purkinje cells. The *rcm* transcript is therefore expressed in the cells that undergo aberrant migration during the period in which these migrations occur. Although the level of *rcm* expression in granule neurons significantly decreased by P21, it was maintained in Purkinje cells and remained detectable in these neurons in the adult brain. Cerebellar expression appeared uniform with respect to rostral/caudal and medial/lateral axes. *rcm* transcripts were also detected in the olfactory bulb and the dentate gyrus of the hippocampus, whereas the pontine and olivary nuclei expressed lower levels of the transcript (Fig. 5a). Obvious abnormalities in these structures were not detected in *rcm* homozygotes.

Genetic studies in *C. elegans* indicate that the UNC-5 receptor interacts with the netrin ligand encoded by *unc-6* (refs 4, 6). Netrins promote outgrowth of rat spinal cord commissural axons *in vitro*¹³. *In vivo*, netrin-1 is not only required for guidance of spinal commissural axons, but also for the formation of the pontine nuclei and several brain commissures, indicating a role for this molecule in both cell migration and axonal guidance in the developing brain¹⁴. The Rcm protein binds netrin-1 *in vitro*⁸. The fact that netrin-1 is expressed during embryonic and postnatal development of the rat cerebellum also supports a role for it as an Rcm ligand *in vivo*¹⁵.

Our results indicate that Rcm protein and its ligand are important in critical migratory and/or cell proliferation events during cerebellar development. The *rcm* mutations only affect the migration of a subset of *rcm*-expressing cells in the developing brain, an

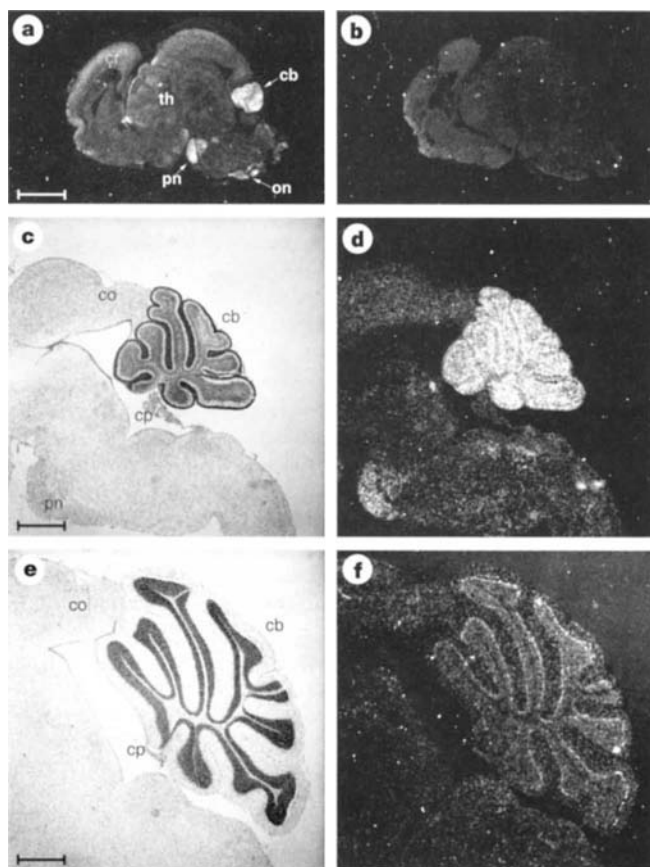


Figure 5 Localization of *rcm* transcript by *in situ* hybridization of mid-sagittal sections of normal neonatal mouse brain. Dark-field images of adjacent sections of P0 brain demonstrate the localization of the signal after hybridization with antisense (a) and the negative control sense (b) strand *rcm* probes. Bright-field (c, e) and corresponding dark-field (d, f) micrographs illustrate the expression pattern of the antisense *rcm* probe in P7 (c, d) and P21 (e, f) cerebellum. Abbreviations: cb, cerebellum; cp, choroid plexus; co, colliculi; cr, cerebral hemisphere; pn, pontine nuclei; on, olivary nuclei; th, thalamus. Scale bars represent 1.25 mm (a, b) and 0.65 mm (c-f).

observation that implies genetic redundancy as the expression domains of *rcm* within the cerebellum and other regions of the developing brain overlap with those of *Unc5 h-1* and *Unc5 h-2*, two other members of the vertebrate UNC-5-like gene family⁸. □

Methods

Mice. *rcm*¹⁸/*rcm*¹⁸ animals were derived from a previously described *Ucp1* transgenic mouse lineage produced and maintained on a C57BL/6J × SJL/J background¹⁶. *rcm*⁵/*rcm*⁵ mice were obtained from the Mouse Mutant Resource at The Jackson Laboratory. This mutation arose on a C57BL/6J background and is currently maintained on an F₁ hybrid B6C3HFe-a/a background.

Histology and immunohistochemistry. For histological examination, 7–10 adult mice were perfused with Bouin's solution, cerebella were removed and then postfixed overnight. Neonatal brains (3–4 at each time point) were immersion-fixed in paraformaldehyde. Slides were examined on a Leica DMRXE microscope and high-resolution digital images were recorded by a Kodak Megaplus 1.4 camera.

Sections were incubated overnight at 4 °C with rabbit polyclonal antiserum against calbindin-D (Swant), diluted 1 : 1,500. Antibody binding was detected using biotinylated anti-rabbit IgG biotin conjugate (Sigma) diluted 1 : 50, and the ExtrAvidin peroxidase staining kit using DAB as enzyme substrate (Sigma).

Genomic library construction and genetic mapping. The genomic DNA library was prepared by ligation and packaging of *MboI* partially digested spleen DNA from a homozygous *rcm*¹⁸ mouse, according to the manufacturer's

protocol. Positive clones were identified by hybridization to a 1.8-kb *PstI/HindIII* fragment of the *Ucp1* transgene¹⁶. P1 clones were isolated from a 129 ES cell library¹⁷.

5' and 3' RACE analysis. PCR reactions were done with Marathon RACE-ready cDNA and KlenTaq Polymerase (Clontech) according to the manufacturer's protocol. Products were ligated into the TA-cloning vector PCR II (Invitrogen), and sequenced on the ABI 373 Stretch DNA sequencer using dideoxylterminator chemistry.

Northern blot analysis. Total RNA was prepared from tissues by the guanidinium isothiocyanate method and poly(A)⁺ RNA was isolated using oligo(dT) cellulose¹⁸. RNA was electrophoresed through formaldehyde gels and transferred to nylon filters (Magna, MSI) by standard methods. Membranes were hybridized and washed as described¹⁹.

In situ hybridization. ³³P-labelled sense and antisense riboprobes (10⁵ c.p.m. μl⁻¹) were generated from a plasmid containing 582 bp of the coding region of the RCM cytoplasmic domain or 1.1 kb of the *Math1* open reading frame. Sample preparation, hybridization, and post-hybridization washes were as described except that hybridizations were done at 50 °C and the initial wash in 50% formamide/2 × SSC was for 2 h at 37 °C²⁰. Slides were dipped in Kodak NTB 2 emulsion, exposed for 24 h at 4 °C, and developed in Kodak D19. Sections were stained with haematoxylin and photographed as described.

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1. Cowan, W. M. *Development in the Nervous System* (Cambridge University Press, London, 1981).
2. Hatten, M. E. & Heintz, N. Mechanisms of neural patterning and specification in the developing cerebellum. *Annu. Rev. Neurosci.* **18**, 385–408 (1995).
3. Lane, P. W., Bronson, R. T. & Spencer, C. A. Rostral cerebellar malformation (*rcm*): A new recessive mutation on chromosome 3 of the mouse. *J. Hered.* **83**, 315–318 (1992).
4. Hedgecock, E. M., Culotti, J. G. & Hall, D. H. The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* **4**, 61–85 (1990).
5. Leung-Hagesteijn, C. et al. UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in *C. elegans*. *Cell* **71**, 289–299 (1992).
6. Hamelin, M., Zhou, Y., Su, M.-W., Scott, I. M. & Culotti, J. G. Expression of the UNC-5 guidance receptor in the touch neurons of *C. elegans* steers their axons dorsally. *Nature* **364**, 327–330 (1993).
7. Wadsworth, W. G., Bhatt, H. & Hedgecock, E. M. Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in *C. elegans*. *Neuron* **16**, 36–46 (1996).
8. Leonardo, E. D. et al. Vertebrate homologues of *C. elegans* UNC-5 are candidate netrin receptors. *Nature* **386**, 833–838 (1997).
9. Akazawa, C. et al. A mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene *atonal* is a positive transcriptional regulator expressed in the developing nervous system. *J. Biol. Chem.* **270**, 8730–8738 (1995).
10. Ben-Arie, N. et al. Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. *Hum. Mol. Gen.* **4**, 1207–1216 (1996).
11. Johnson, K. R., Cook, S. A. & Davisson, M. T. Chromosomal localization of the murine gene and two related sequences encoding high-mobility-group 1 and Y proteins. *Genomics* **12**, 503–509 (1992).
12. Willott, E. et al. The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions. *Proc. Natl Acad. Sci. USA* **90**, 7834–7838 (1993).
13. Serafini, T. et al. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78**, 409–424 (1994).
14. Serafini, T. et al. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001–1014 (1996).
15. Livesey, F. J. & Hunt, S. P. Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral and cerebellar development. *Mol. Cell. Neurosci.* (in the press).
16. Boyer, B. B. & Kozak, L. P. The mitochondrial uncoupling protein gene in brown fat: Correlation between DNase I hypersensitivity and expression in transgenic mice. *Mol. Cell. Biol.* **11**, 4147–4156 (1991).
17. Sternberg, N., Smoller, D. & Braden, T. Three new developments in P1 cloning. Increased cloning efficiency, improved clone recovery, and a new P1 mouse library. *Genet. Anal. Tech. Appl.* **11**, 171–80 (1994).
18. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156–159 (1987).
19. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).
20. Hui, C.-C. & Joyner, A. L. A mouse model of Greig cephalo-polysyndactyly syndrome: the *extra-toes* mutation contains an intragenic deletion of the *Gli3* gene. *Nature Genet.* **3**, 241–245 (1993).
21. Kyte, J. & Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132 (1982).
22. von Heijne, G. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683–4690 (1986).
23. Klein, P., Kanehisa, M. & Delisi, C. The detection and classification of membrane-spanning proteins. *Biochim. Biophys. Acta* **815**, 468–476 (1985).
24. Singer, S. F. The structure and insertion of integral proteins in membranes. *Annu. Rev. Cell Biol.* **6**, 247–296 (1990).

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Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion

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Integrins are important mediators of cell adhesion to extracellular ligands and can transduce biochemical signals both into and out of cells^{1,2}. The cytoplasmic domains of integrins interact with several structural and signalling proteins and consequently participate in the regulation of cell shape, motility, growth and differentiation³. It has been shown that calreticulin associates with the cytoplasmic domains of integrin α -subunits and that this interaction can influence integrin-mediated cell adhesion to extracellular matrix^{4,5}. We have now developed calreticulin-deficient embryonic stem (ES) cells and isolated embryonic fibroblasts from calreticulin mutant mice. We find that in both cell types integrin-mediated adhesion is severely impaired, although integrin expression is unaltered. Expression of recombinant calreticulin in double knockout ES cells by complementary DNA transfection rescued integrin-mediated adhesion. In wild-type cells, engagement of surface integrins induced a transient elevation in cytosolic calcium concentration owing to influx of extracellular calcium. This calcium transient was absent in calreticulin-deficient cells. In contrast, the amount of calcium in endomembrane stores, which is sensitive to both inositol 1,4,5-trisphosphate and thapsigargin, was indistinguishable in the two cell types. Our results indicate that calreticulin is an essential modulator both of integrin adhesive functions and integrin-initiated signalling, but that it may not play a significant role in the storage of luminal calcium.

ES cells targeted at one calreticulin allele were engineered by inserting the PGKneo⁶ selection cassette in the antisense orientation at the calreticulin start site through homologous recombination (Fig. 1a)⁷. ES cells homozygous for the calreticulin mutation (double knockout) were isolated by culturing calreticulin-targeted ES cells in increased G418 concentrations. The heterozygous and homozygous genotypes were demonstrated by Southern blotting (Fig. 1b and c, respectively) and the null calreticulin phenotype was confirmed by immunoblotting with calreticulin antibodies (Fig. 1d). Three clones of ES cells were used extensively in our experiments: parental cells (WT), a heterozygous clone (clone 34) and a homozygous clone (clone 29).

The growth rates of these ES cell clones were assessed in medium containing 10% serum and were all found to be similar (data not shown). Their adhesive properties were assessed by quantitative cell attachment assay on fibronectin and laminin under serum-free conditions. Wild-type cells adhered to fibronectin and laminin, whereas adhesion of the cells lacking calreticulin (clone 29) was significantly reduced on these substrates (Fig. 2a). ES cells lacking calreticulin were also impaired in their ability to adhere to anti- $\alpha_5\beta_1$ integrin antibodies (Fig. 2a). The heterozygous clone 34 was generally intermediate in its adhesive capacity. None of the clones tested adhered to vitronectin (Fig. 2a). Wild-type ES cell adhesion to

proteins of the extracellular matrix (ECM) was mediated by integrin because GRGDSP (single-letter amino acid notation) peptide and a function-blocking polyclonal anti-fibronectin-receptor serum inhibited adhesion to fibronectin, and cell attachment to laminin was inhibited by a monoclonal antibody (GoH3) to the integrin α_6 subunit (Fig. 2b). To ensure that these differences in cell adhesion were not due to differences in the expression of the relevant integrins, the cell-surface levels of $\alpha_5\beta_1$, α_4 , α_6 and $\alpha_v\beta_3$ integrins were assessed by flow cytometry. All clones tested expressed similar amounts of integrin subunits (Fig. 2c).

We also isolated fibroblasts from mouse embryos lacking calreticulin (details of the mouse phenotype will be published elsewhere). The knockout fibroblasts had markedly impaired integrin-mediated adhesion to fibronectin and vitronectin without altered expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (see Supplementary Information).

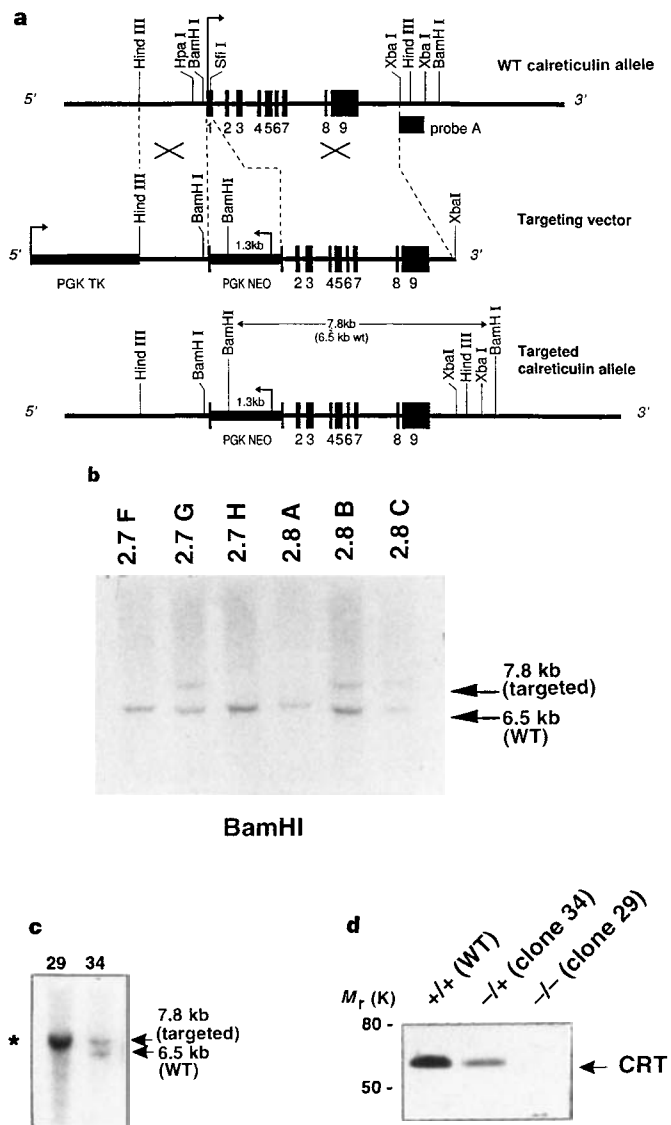


Figure 1 Targeted inactivation of the calreticulin gene in embryonic stem (ES) cells. **a**, The structure of the wild-type allele, targeting vector, and targeted allele are shown together with the pertinent restriction sites. **b**, Southern blot showing three targeted ES cell clones in which one calreticulin allele is disrupted. These heterozygous clones were inoculated into mouse blastocysts to generate calreticulin-null mice. **c**, Southern blot of double knockout ES cell clones. Asterisk indicates the twice-targeted calreticulin alleles. **d**, Western blot of wild-type, heterozygous (one allele targeted, number 34) and homozygous (both alleles targeted, no. 29) ES cell clones. Coomassie-blue staining of the membrane demonstrated equal protein loading in all lanes (not shown). CRT, calreticulin.

To confirm a role for calreticulin in modulating integrin function in ES cells, we introduced calreticulin into calreticulin-negative ES cells (clone 29) by transient transfection with the pRC/CMV expression vector containing a 1.8-kilobase (kb) full-length calreticulin cDNA. As a control, transfections were also carried out with pRC/CMV containing calreticulin cDNA in the antisense orientation. Calreticulin expression in clone 29 transfectants was confirmed by immunoblotting (Fig. 3a). These cells demonstrated significantly increased adhesion to fibronectin compared to antisense-transfected cells in which there was no calreticulin expression (Fig. 3b).

As calreticulin has been implicated in the regulation of calcium signalling, especially in the endoplasmic reticulum⁸⁻¹⁰, we examined calcium regulation in the wild-type and calreticulin-knockout ES cells. The resting cytosolic calcium concentration ($[Ca^{2+}]_i$) in parental and calreticulin-deficient cells was similar. More important, the cytosolic $[Ca^{2+}]_i$ transient elicited by addition of thapsigargin to cells bathed in Ca^{2+} -free medium (Fig. 4a), which is a measure of the calcium stored in the endoplasmic reticulum, was also indistinguishable in the two cell types (Fig. 4a). Subsequent addition of extracellular calcium resulted in a large and sustained

increase in cytosolic $[Ca^{2+}]_i$ (Fig. 4a). This increase, which is due to the opening of plasmalemmal channels in response to the depletion of endomembrane stores, was comparable in wild-type and calreticulin-deficient cells (Fig. 4a). Because the cytosolic calcium-buffering power of the two cell types (determined by loading the cytosol with varying doses of an exogenous calcium chelator¹¹) was similar (Fig. 4c), these findings indicate that calreticulin is probably not required to signal capacitative calcium influx across the plasma membrane and that it is not critical for luminal calcium storage in the endoplasmic reticulum of ES cells.

Calreticulin can regulate inositol 1,4,5-trisphosphate ($InsP_3$)-mediated calcium signalling in *Xenopus* oocytes⁹. To examine the role of calreticulin in the storage and mobilization of calcium from $InsP_3$ -sensitive stores, we challenged ES cells with lysophosphatidic acid (LPA, 100 nM), which induces $InsP_3$ release¹² through heterotrimeric G-protein-mediated activation of phospholipase C. As shown in Fig. 4b, when added to cells in Ca^{2+} -free medium, LPA induced a rapid $[Ca^{2+}]_i$ transient. The magnitude of this transient was indistinguishable in parental and clone 29 cells (Fig. 4b). Subsequent addition of thapsigargin induced a second, smaller

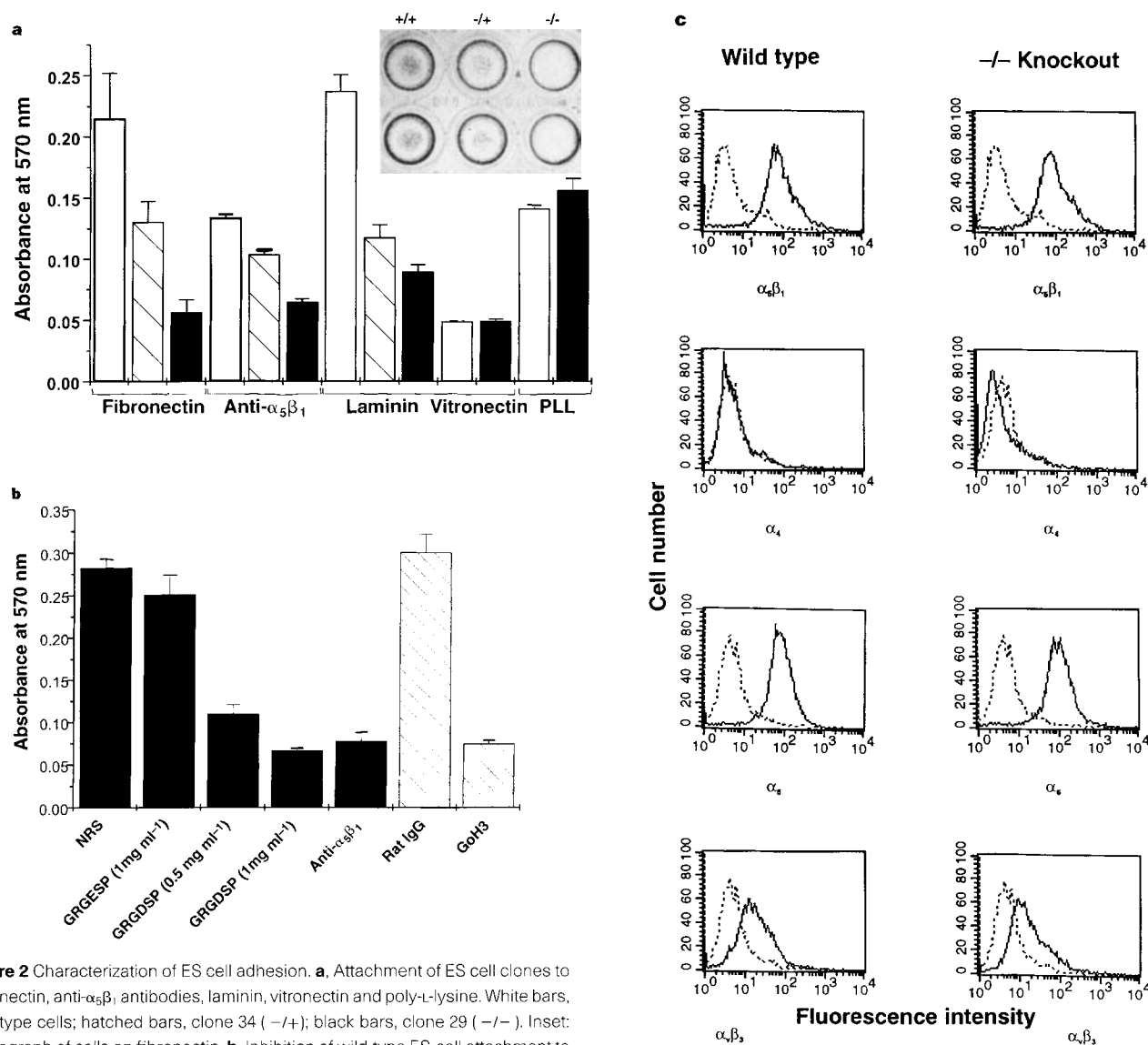


Figure 2 Characterization of ES cell adhesion. **a**, Attachment of ES cell clones to fibronectin, anti- $\alpha_5\beta_1$ antibodies, laminin, vitronectin and poly-L-lysine. White bars, wild-type cells; hatched bars, clone 34 (-/+); black bars, clone 29 (-/-). Inset: photograph of cells on fibronectin. **b**, Inhibition of wild-type ES-cell attachment to fibronectin and laminin. Antisera were used at 10 μ g ml⁻¹. NRS, normal rabbit serum. Black bars, attachment to fibronectin; hatched bars, attachment to laminin. In **a** and **b**, means and s.e.m. are calculated from at least three independent experiments. **c**, Quantification of cell-surface integrin expression by flow cytometry of wild-type and clone 29 (-/-) ES cells.

burst of net Ca^{2+} release which was considerably smaller than that shown in Fig. 4a, implying that the stores depleted by LPA are a subset of the thapsigargin-sensitive compartment. The amount of Ca^{2+} released by thapsigargin after LPA was not significantly different in parental and calreticulin-null cells (Fig. 4b), suggesting that calreticulin is not required for the initial InsP_3 -sensitive Ca^{2+} release; although the decay phase of Ca^{2+} release in clone 29 cells is slower than in ES cells (Fig. 4b), this was not consistent in all cells. Our findings disagree with a previous report in which the Ca^{2+} response to bradykinin was diminished as a result of antisense oligonucleotide downregulation of calreticulin expression¹³. However, our data clearly show that Ca^{2+} storage in the endoplasmic reticulum and InsP_3 -mediated Ca^{2+} release are unaffected in the absence of calreticulin. Although our results are in partial agreement with those of Bastianutto *et al.*¹⁴, who found that the level of calreticulin had no effect on the initial peak of InsP_3 -sensitive Ca^{2+} release, these investigators did find that subsequent oscillations of Ca^{2+} could be affected by overexpression of calreticulin. Our results are also broadly consistent with those of Camacho and Lechleiter⁹, who found that InsP_3 -mediated Ca^{2+} release could be regulated by calreticulin but that this function did not correlate with its capacity to store Ca^{2+} , suggesting that calreticulin has a signalling function in Ca^{2+} metabolism and does not just sequester it.

One of the earliest responses to integrin-mediated cell spreading is a stimulation of Ca^{2+} influx^{15,16}. Because ES cells lacking calreticulin do not adhere and spread normally on ECM substrates, we examined integrin-mediated calcium signalling by monitoring the fluorescence of Fura-2-loaded cells as they interacted with glass coverslips coated with either an anti- $\alpha_5\beta_1$ integrin antibody or with fibronectin (Fig. 4d). In most of the wild-type cells, engagement of surface integrins induced an increase in cytosolic $[\text{Ca}^{2+}]$ (see Fig. 4d for example) that was often accompanied by visible spreading of the cells on the substratum. This rise was primarily due to influx of extracellular Ca^{2+} , as it did not occur when cells attached in Ca^{2+} -free medium ($n = 12$; data not shown). By contrast, nearly all calreticulin-null cells failed to spread and no changes in cytosolic $[\text{Ca}^{2+}]$ were detected (Fig. 4d).

A transient increase in cytosolic $[\text{Ca}^{2+}]$ shortly after integrin ligand binding may be important in normal receptor post-occupation events (such as increased avidity, cytoskeletal reorganization). We therefore tested whether stimulation of downstream events in integrin 'outside-in' signalling within calreticulin-null cells could compensate for their adhesion defect. We found that when calreticulin knockout cells were treated with 100 nM LPA their adhesion to fibronectin was significantly restored (Fig. 4e). Although not shown in Fig. 4, the response to LPA was dose-dependent. Similar results were obtained when the calreticulin^{-/-} cells were treated with thapsigargin (Fig. 4e). Together these results suggest that stimulation of intracellular signalling events downstream of integrin occupancy, possibly through a transient increase in cytosolic $[\text{Ca}^{2+}]$ alone, can compensate for the loss of calreticulin-dependent adhesion.

Calreticulin is known to regulate calcium concentration in the endoplasmic reticulum^{8,9} and to associate with proteins in the cytoplasm, nucleus and extracellular compartments¹⁷⁻²⁰. We have now provided new insight into the cellular functions of calreticulin. We conclude that (1) calreticulin is essential for normal, integrin-mediated adhesion to extracellular matrix substrates; (2) the calcium storage functions of calreticulin are not essential; (3) lack of calreticulin perturbs integrin-mediated influx of extracellular Ca^{2+} , possibly contributing to the loss of integrin-mediated cell adhesion; in calreticulin-null cells integrin ligand binding *per se* may not be impaired and decreased adhesion may follow from the perturbation of early downstream signals such as Ca^{2+} influx. Our finding that LPA can compensate for lack of calreticulin supports this idea. Although it has been suggested that calreticulin may influence cell adhesion through alteration in vinculin expression²¹, we have

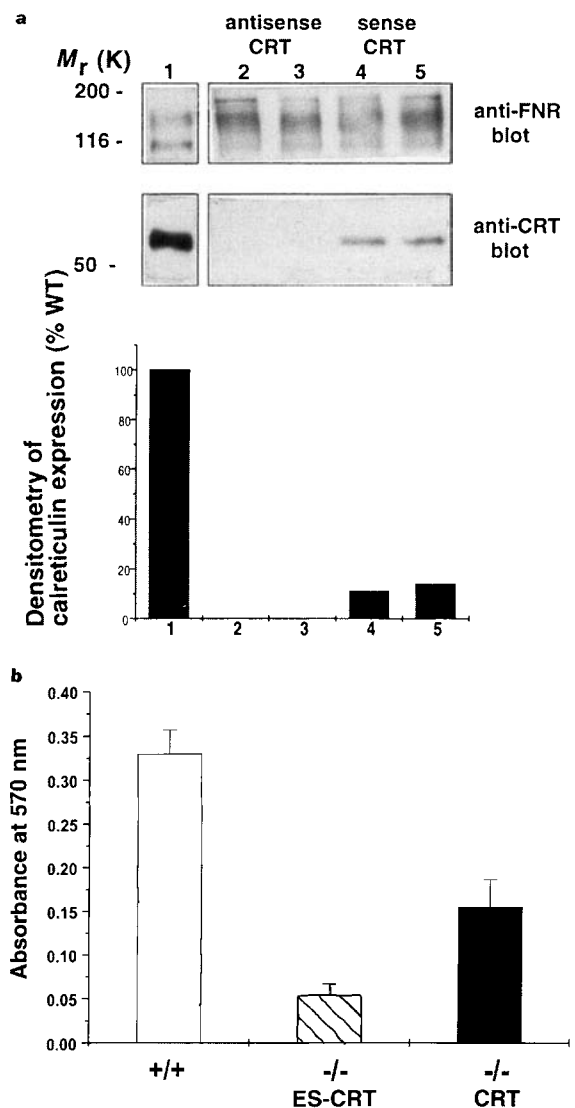


Figure 3 Rescue of knockout ES cell adhesion by transfection with calreticulin (CRT). **a**, Expression of calreticulin in ES cell clone 29 by transfection with pRC/CMV-calreticulin. Western blot of cell lysates from 4 independent transfections is shown (bottom panel), with the corresponding anti-fibronectin-receptor blot (top). Lanes: 1, wild-type ES cell lysate; 2 and 3, clone 29 cells transfected with antisense calreticulin; 4 and 5, clone 29 cells transfected with sense calreticulin. Bottom, densitometer quantification of calreticulin expression per μg lysate. **b**, Attachment of ES cell clone 29 transfectants to fibronectin. White bar, wild-type ES cells; hatched bar, ES cell clone 29 transfected with antisense calreticulin (ES-CRT); black bar, ES cell clone 29 transfected with sense calreticulin (CRT). Means and standard errors are cumulated from 5 independent experiments. The attachment of CRT transfectants is significantly greater ($P < 0.05$) than that of AS-CRT transfectants.

confirmed that there is no difference in vinculin expression in wild-type or calreticulin^{-/-} ES cells (data not shown). We have shown that calreticulin is essential for the normal function of several integrin receptors, including in early integrin-mediated 'outside-in' signalling. Calreticulin-null cells will also be useful in the investigation of other postulated roles of calreticulin such as the modulation of steroid hormone receptor function²²⁻²⁴.

Methods

Targeting vector. Full-length 1.9-kb human calreticulin cDNA²⁵ was used as a probe to isolate a 14-kb murine calreticulin genomic clone from a library constructed from DNA of the 129 Sv strain (Stratagene). The PGKneo selection cassette²⁶ was inserted in the opposite orientation at the unique *Sfi*I site

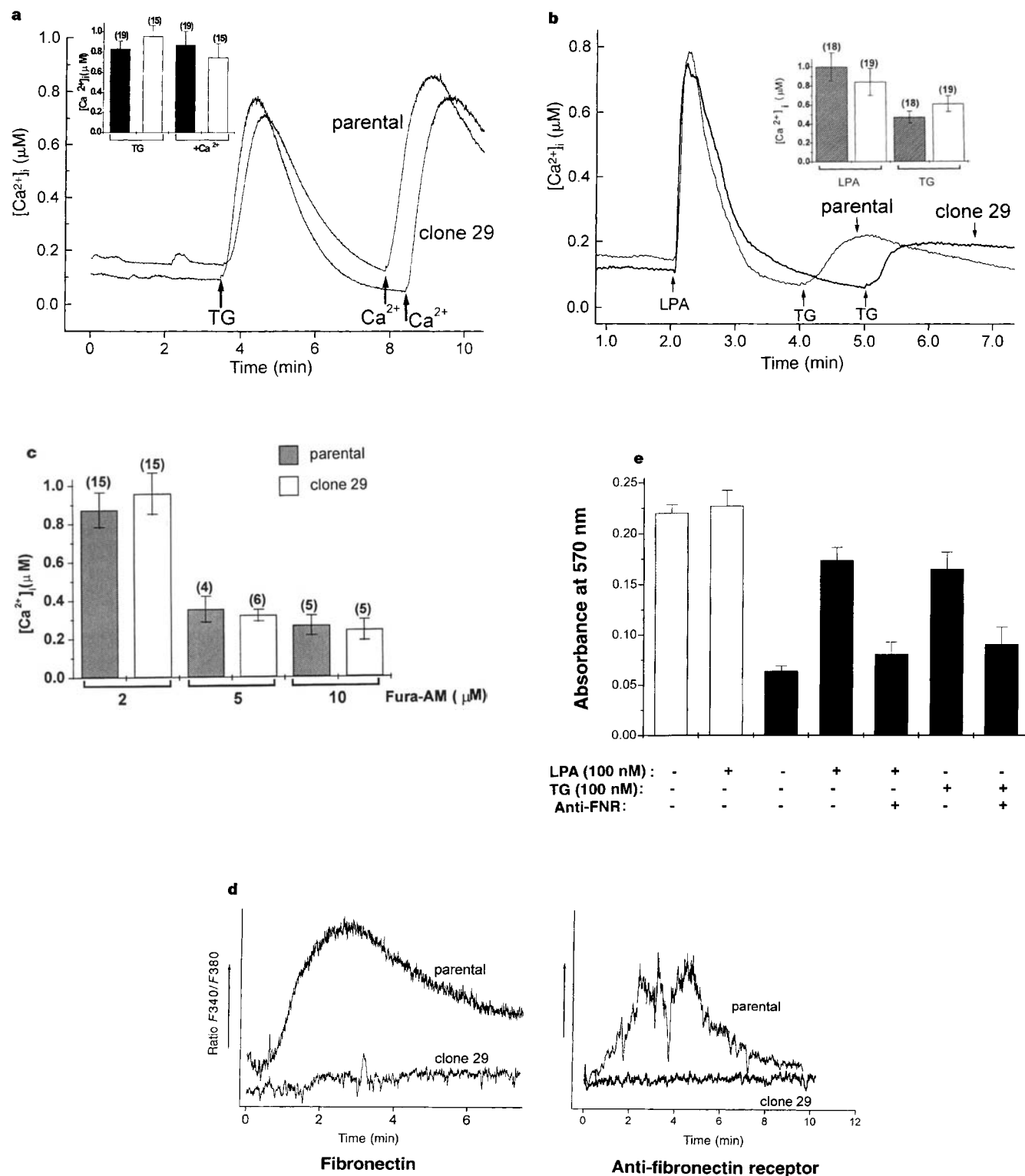


Figure 4 Assessment of calcium storage in endomembrane compartments and of integrin-mediated cytosolic $[Ca^{2+}]$ transients in ES cells. **a**, Cytosolic $[Ca^{2+}]$ in adherent ES cells. Where indicated, thapsigargin (TG, 100 nM) and subsequently 1 mM free calcium were added to the medium. Representative traces are shown. Inset: average peak $[Ca^{2+}]$, attained in parental (stippled bar; $n = 19$) or knockout (clone 29, open bar; $n = 15$). **b**, As **a**, except that the indicated cells were stimulated with lysophosphatidic acid (LPA, 100 nM) followed by thapsigargin. Inset: average peaks of $[Ca^{2+}]$, attained in parental (stippled bar; $n = 18$) and knockout cells (clone 29, open bar; $n = 19$). **c**, Comparison of the cytosolic calcium-buffering power in parental and knockout ES cells. The peak $[Ca^{2+}]$,

induced by thapsigargin was measured in cells incubated with the indicated concentrations of the acetoxymethyl (AM) ester precursor of Fura-2. The number of experiments is indicated in parentheses. **d**, Transient increases in $[Ca^{2+}]$ in ES cells plated on fibronectin (representative of 9 wild-type and 9 knockout cells) or anti-fibronectin-receptor antiserum (representative of 16 wild-type and 9 knockout cells). **e**, Adhesion assays to fibronectin, as shown in Fig. 2. Where indicated, 100 nM LPA or 100 nM TG and anti-FNR antiserum ($10 \mu\text{g ml}^{-1}$) were added. White bars, parental ES cells; black bars, knockout ES cells. All data are means $\pm$ s.e.m. of at least 3 experiments.

immediately downstream of the translation start site by blunt-end cloning. The PGKtk cassette²⁶ was then cloned at the 5' HindIII site. The resulting targeting vector featured 2 kb of homologous sequence on the short arm and 5 kb on the long arm (Fig. 1a).

ES cell culture and transfection. The R1 ES cell line was cultured and electroporated as described²⁷. Colonies were picked after 8 days in selection medium and expanded²⁷. For isolating cells homozygous for the targeted allele, the heterozygous clone 2F (clone 34) was grown on feeder cells in 4 mg ml⁻¹ G418 (ref. 28). Resistant colonies were picked after 9 days in high-concentration selection medium. To obtain expression of calreticulin in homozygous mutant ES cells, the pRC/CMV expression vector containing a 1.8-kb full-length calreticulin cDNA in either the sense or the antisense orientation²³ was transiently transfected into ES cell clone 29 using lipofectin according to the manufacturer's instructions (Gibco/BRL).

Southern blot analysis. DNA was isolated from cultured cells as described²⁷ and tested for integration at the targeted locus by Southern blotting of BamHI digests. The probe was a 1-kb XbaI genomic fragment located immediately downstream of the region of homology of the targeting vector.

Attachment assays and analysis of integrin expression. Serum-free adhesion assays are described elsewhere⁵. 0.5–1 mg ml⁻¹ GRGDSP or GRGESP peptide (Gibco/BRL), 10 µg ml⁻¹ rabbit anti-fibronectin receptor (Gibco/BRL) or 10 µg ml⁻¹ rat anti-α₆ (GoH3; AMAC) was added where indicated. For flow-cytometric analysis of integrin expression, 1 × 10⁶ cells were incubated on ice for 30 min with primary antibody: anti-FNR, anti-VNR (Gibco/BRL), anti-α₆ (GoH3) or anti-α₄ (ref. 29) in PBS, 0.1% sodium azide, 2.0% fetal bovine serum. Cells were washed and then incubated for 30 min with secondary antibody: FITC-conjugated goat anti-rabbit or FITC-conjugated goat anti-rat (Jackson ImmunoResearch). Cells were fixed in PBS, 1.0% paraformaldehyde, pelleted, resuspended in PBS and analysed on a FACScan (Becton Dickinson Immunocytometry Systems).

Cytosolic calcium measurements. To measure cytosolic free calcium, cells were loaded with Fura-2 by incubation with 0.5–10 µM of the precursor acetoxymethyl ester for 20 min at 37 °C. Fura-2 ratio fluorescence measurements were made on a Nikon Diaphot TMD microscope (Nikon) equipped with a 100 W Xenon lamp, a shutter, rotating mirror, fibre optic assembly (RatioMaster, Photon Technologies), a Fluor 40 × 1.3 oil-immersion objective and a high-sensitivity photometer (D-104, PTI) interfaced to a 386 computer (NEC) via a 12 bit A/D board (Labmaster, National Instruments). The cells were alternately excited at 340 and 380 nm while recording the emission at 510 nm. Photometric data were acquired at 10 Hz using Oscar software (PTI). The microscope was equipped with a separate light source for long-wavelength transillumination (λ > 620 nm), a 580-nm emission dichroic mirror and a separate video camera (MTI 72, Dage-MTI) to allow continuous Hoffmann-enhanced visualization of the cells during fluorescence measurements. Cytosolic calcium buffering power was estimated by measuring the magnitude of calcium transients in cells loaded with increasing concentrations Fura-2 (ref. 11). The intracellular concentration of Fura-2 was measured at the isosbestic wavelength (360 nm) and compared with standards. Unless indicated otherwise, all measurements of cytosolic [Ca²⁺] were made in Ca²⁺-free medium. To assess integrin-mediated Ca²⁺ influx, coverslips were coated with polyclonal anti-α₅β₁ antiserum or with fibronectin (each at 10 µg ml⁻¹). In medium containing 1 mM Ca²⁺, suspended ES cells were allowed to sediment onto the coverslips at [Ca²⁺]_i and cell morphology were monitored continuously and simultaneously. Traces start at the time of contact of a single cell with the coated coverslip.

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- Hynes, R. O. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell* **69**, 11–25 (1992).
- Schwartz, M. A., Schaller, M. D. & Ginsberg, M. H. Integrins: Emerging paradigms of signal transduction. *Annu. Rev. Cell Biol. and Dev. Biol.* **11**, 549–600 (1995).
- Dedhar, S. & Hannigan, G. E. Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr. Opin. Cell Biol.* **8**, 657–669 (1996).
- Coppolino, M. G. et al. Inducible interaction of integrin α₅β₁ with calreticulin: dependence on the activation state of the integrin. *J. Biol. Chem.* **270**, 23132–23138 (1995).
- Leung-Hagstjorn, C. et al. Cell attachment to extracellular matrix substrates is inhibited upon downregulation of expression of calreticulin, an intracellular integrin α-subunit-binding protein. *J. Cell. Sci.* **107**, 589–600 (1994).
- Mansour, S. L. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy of targeting mutations to non-selectable genes. *Nature* **336**, 348–352 (1988).
- Capecchi, M. The new mouse genetics: altering the genome by gene targeting. *Trends Genet.* **5**, 70–76 (1989).
- Michalak, M. et al. Calreticulin. *Biochem. J.* **285**, 681–692 (1992).

- Camacho, P. & Lechleiter, J. D. Calreticulin inhibits repetitive intracellular Ca²⁺ waves. *Cell* **82**, 765–771 (1995).
- Mery, L. et al. Overexpression of calreticulin increases intracellular Ca²⁺ storage and decreases store-operated Ca²⁺ influx. *J. Biol. Chem.* **271**, 9332–9339 (1996).
- von Tscharn, V., Deranleau, D. A. & Baggiolini, M. Calcium fluxes and calcium buffering in human neutrophils. *J. Biol. Chem.* **261**, 10163–10168 (1986).
- Jalink, K. et al. Lysophosphatidic acid-induced Ca²⁺ mobilization in human A431 cells: structure-activity analysis. *Biochem. J.* **307**, 609–616 (1995).
- Liu, N. et al. Decreasing calreticulin expression lowers the Ca²⁺ response to bradykinin and increases sensitivity to ionomycin in NG-108-15 cells. *J. Biol. Chem.* **269**, 28635–28639 (1994).
- Bastianutto, C. et al. Overexpression of calreticulin increases the Ca²⁺ capacity of rapidly exchanging Ca²⁺ stores and reveals aspects of their luminal microenvironment and function. *J. Cell Biol.* **130**, 847–855 (1995).
- Schwartz, M. A. Spreading of human endothelial cells on fibronectin or vitronectin triggers elevation of intracellular free calcium. *J. Cell Biol.* **120**, 1003–1010 (1993).
- Sjaastad, M. D., Lewis, R. S. & Nelson, W. J. Mechanisms of integrin-mediated calcium signaling in MDCK cells: regulation of adhesion by IP₃- and store-independent calcium influx. *Mol. Biol. Cell* **1**, 1025–1041 (1996).
- Dedhar, S. Novel functions for calreticulin: interaction with integrins and modulation of gene expression? *Trends Biochem. Sci.* **19**, 269–271 (1994).
- Sontheimer, R. D. et al. The unveiling of calreticulin—a clinically relevant tour of modern cell biology. *J. Invest. Med.* **43**, 362–370 (1995).
- Rojiani, M. V. et al. In vitro interaction of a polypeptide homologous to human R0/SS-A antigen (calreticulin) with a highly conserved amino-acid sequence in the cytoplasmic domain of integrin α-subunits. *Biochemistry* **30**, 9859–9866 (1991).
- Williams, M. J. et al. The inner world of cell adhesion: integrin cytoplasmic domains. *Trends Cell Biol.* **4**, 109–112 (1994).
- Opas, M. et al. Calreticulin modulates cell adhesiveness via regulation of vinculin expression. *J. Cell Biol.* **135**, 1913–1923 (1996).
- Burns, K. et al. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* **367**, 476–480 (1994).
- Dedhar, S. et al. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* **367**, 480–483 (1994).
- St Arnaud, R. et al. Constitutive expression of calreticulin in osteoblasts inhibits mineralization. *J. Cell Biol.* **131**, 1351–1359 (1995).
- McCauliffe, D. P. et al. Molecular cloning, expression, and chromosome 19 localization of a human R0/SS-A autoantigen. *J. Clin. Invest.* **86**, 332–335 (1990).
- Rudnicki, M. A. et al. Inactivation of MyoD in mice leads to upregulation of the myogenic H1H gene myf-5 and results in apparently normal muscle development. *Cell* **71**, 383–390 (1992).
- Hogan, B. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, New York, 1994).
- Mortensen, R. M. Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell Biol.* **12**, 2391–2395 (1992).
- Miyake, K. et al. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* **173**, 599–607 (1991).

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Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy

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Progressive myoclonus epilepsy of the Unverricht–Lundborg type (EPM1; MIM 254800) is an autosomal recessive disorder with onset between 6 and 13 years followed by variable progression to mental deterioration and cerebellar ataxia¹. It is a rare disorder but more common in Finland (1 in 20,000) and the western Mediterranean^{1,2}. Two point mutations in the cysteine proteinase inhibitor gene cystatin B (CSTB), proved that this gene is responsible for EPM1 (ref. 3). An extensive search in the CSTB gene revealed mutations accounting only for 14% of the 58 unrelated

EPM1 alleles studied⁴. Here we report that the majority of EPM1 alleles contain expansions of a dodecamer (12-mer) repeat located about 70 nucleotides upstream of the transcription start site nearest to the 5' end of the CSTB gene. Normal alleles contain 2 or 3 copies of this repeat whereas mutant alleles contain more than 60 such repeats and have reduced levels of CSTB messenger RNA in blood but not in cell lines. 'Premutation' CSTB alleles with 12–17 repeats show marked instability when transmitted to offspring.

Southern blot analysis of DNAs from EPM1 patients with unknown CSTB mutations that we found in our previous analysis⁴, and of DNA from controls, revealed that the *EcoRI* fragment, which is normally 9 kilobases (kb) long and contains the entire CSTB gene, was larger in these patients (Fig. 1a, b). This larger fragment was not present in any of the 50 normal alleles tested. Heterozygous carrier parents showed one larger allele that was of similar size to that of their affected children. Thus, larger *EcoRI* fragments (but of varying sizes between patients) segregate with EPM1 in many families. Moreover, all the EPM1 patients with a known mutation in one allele⁴ were heterozygous for the normal and a larger *EcoRI* fragment. Southern blot analysis with several enzyme/probe combinations and polymerase chain reaction (PCR) amplification with numerous pairs of oligonucleotide primers precisely localized a genomic region demarcated by oligonucleotide primers 8L and 10R that never resulted in amplification from DNAs homozygous for abnormally large CSTB alleles, whereas all the other PCRs resulted in amplified products of the expected size (Fig. 1c). Thus, an abnormality between nucleotides -400 and -80 upstream of the ATG translation initiation codon of CSTB was probably the cause of the most common mutation in EPM1 alleles. We have previously reported that the dodecamer CCCC GCCCGCG, which is located within this area, is polymorphic and repeated 2 or 3 times in tandem in normal alleles⁴.

To clone and sequence the abnormal fragments, we constructed genomic *EcoRI*–*HindIII* libraries in pBluescript from two patients (see Methods). Appropriate clones were obtained from both DNAs although we noticed that the cloned DNAs were unstable in bacteria and had a high frequency of insert loss. Sequencing of the ends of the cloned *EcoRI*–*HindIII* fragments revealed no genomic rearrangement, translocation or new sequences. Consistent with the region of EPM1 alleles that were refractory to PCR, it was difficult to obtain the nucleotide sequence between nucleotides -210 and -174 upstream of the ATG translation initiation codon (Fig. 1a). The combination of either *Pfu* polymerase or ThermoSequenase with

15–20% dimethylsulphoxide (DMSO) and use of the oligonucleotide primer 10R resulted in the demonstration of a long stretch of uninterrupted perfect repetition of the dodecamer CCCC GCCCGCG sequence (Fig. 2). An expansion to more than 50 repeats was observed in clones from both patients. Because the difference in size between the normal and the abnormal fragments was about 0.8 to 1.5 kb in different patients we concluded that there were more than 60 copies of the repeat in the affected alleles.

Southern blot and/or PCR analysis revealed that 50 of the 58 unrelated EPM1 alleles in the families studied contained an expanded dodecamer repeat. All eight EPM1 alleles with deleterious point mutations were shown not to contain the dodecamer expansion and had, as does the normal population, two or three copies of the repeat. These data indicate that the common mutation mechanism in EPM1 is the expansion of the dodecamer repeat about 200 nucleotides upstream of the initiation codon of the CSTB gene. The common Finnish EPM1 mutation is almost certainly due to the dodecamer repeat expansion, as larger fragments were also recently observed in these patients⁵. Moreover, the paternally derived EPM1 allele of patient GVA04 is of Finnish origin and showed a repeat expansion (Fig. 1b). The polymorphic haplotype around this allele is compatible with the common Finnish EPM1 haplotype^{4,6}.

In order to estimate the polymorphic variability of the CCCC GCCCGCG dodecamer in the 5' flanking region of the CSTB gene we examined by PCR the DNA of 75 unrelated CEPH parents⁷ (excluding families 102 and 104, see below), 24 normal blood donors from Valais, Switzerland and 16 normal individuals from North Africa. We exclusively observed alleles with two or three copies of the repeat. The allele with two copies was found in 34% and that with three copies in 66% of the 198 alleles examined in the CEPH parents and Swiss individuals. The frequencies were slightly different in the North Africans (47% for the 2 and 53% for the 3 copies). Statistical analysis showed that the number of heterozygotes was as expected from the Hardy–Weinberg equilibrium.

However, we did observe three alleles with 12 and one with 13 repeat copies in the parents of two CEPH collection pedigrees (102 and 104) from Venezuela (Fig. 3). Further genotyping in these two families revealed that the alleles with 12 or 13 copies were often transmitted unstably from parents to offspring. From 21 transmissions of these alleles we observed six repeat expansions (29%) to alleles with 13, 14, 15 or 17 repeats (Fig. 3). The instability of the 'premutation' alleles of the dodecamer repeat in this family is 29×10^{-2} , whereas the mutation rate of some so-called hypermutable polymorphisms is 0.6×10^{-2} (ref. 8). Using haplotypes of

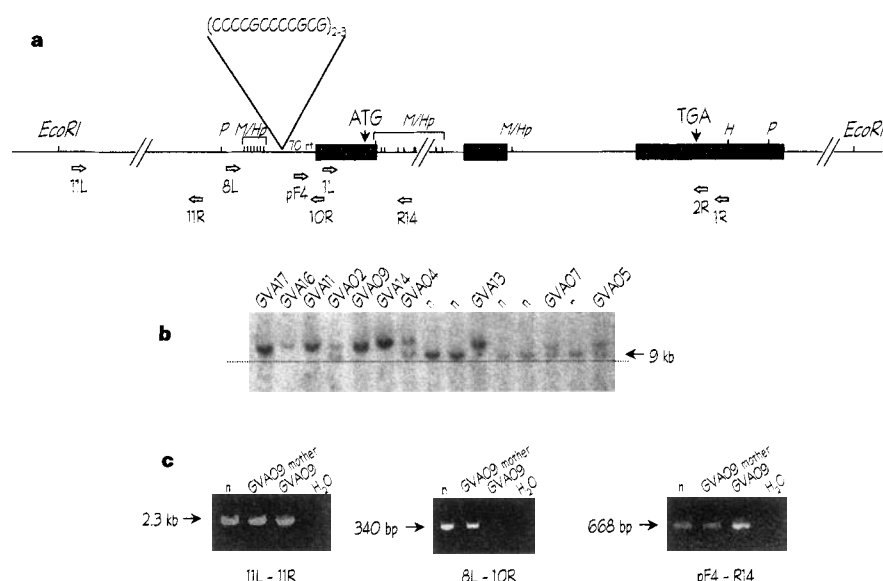


Figure 1 a, Schematic representation of the CSTB gene structure and the dodecamer repeat. The position of the oligonucleotide primers are shown as arrows below the gene. Restriction enzyme sites used in Southern blot and methylation analyses are H: *HindIII*, P: *PstI*, Hp: *HpaI*, M: *MspI*. b, Southern blot analysis of EPM1 patients (GVA) and normals (n). DNA was digested with *EcoRI* and probed with the CSTB cDNA. The normal fragment is 9 kb. Patients GVA17, 16, 11, 09, 14 and 13 show only abnormal (larger) fragments of various sizes. Patients GVA02, 04, 07 and 05, which show two different fragments, all contain defined point mutations in one CSTB allele. The point mutations in all of these patients occurred in the alleles with the normal size fragment. c, Representative PCR amplifications from selected genomic regions. The oligonucleotide primers used are shown below each panel. n, Normal; GVA09, patient homozygous for an abnormal *EcoRI* fragment (see b). The middle panel shows the absence of amplification of the genomic fragment that contains the dodecamer repeat.

informative DNA polymorphisms linked to the CSTB gene (D21S1903, D21S1261, D21S1225, D21S1259, CBS; all within a genetic distance of less than 2 cM from the gene) we determined that all six expansions were transmitted from paternal meioses. The 12-repeat allele expanded in 3 of 18 transmissions (13 maternal and 5 paternal), and the 13-repeat allele expanded in all three transmissions (all paternal). The largest observed expansion was four repeats (from 13 to 17 in the transmission from individual 16 to 23 of Fig. 3a). In addition, haplotype data showed that there were no contractions of the premutation alleles. These data suggest that the alleles with 12 and more repeats may represent 'premutations' of the larger, deleterious expansions. Two offspring from CEPH family 102 were homozygous for 12 copies and one from family 104 was compound heterozygous for 12 and 17 repeats (Fig. 3). These were individuals with no clinical phenotype of EPM1 and therefore these alleles are considered non-causative of EPM1, albeit in the premutation size range.

The dodecamer expansion was observed in EPM1 patients of different geographical origins. The differences of haplotypes of DNA polymorphisms near the CSTB gene⁹ suggest that the expansion mutations had multiple independent origins. We did not observe differences in the expanded alleles between EPM1 patients and their patients, at the level of resolution of the Southern blots. In addition, we found no evidence for somatic mosaicism of the expanded allele.

More than 10 neurological and neuromuscular human disorders

with trinucleotide repeat expansion have been discovered in the past 6 years¹⁰⁻¹³. EPM1 is the second autosomal recessive neurodegenerative disease after Friedreich's ataxia¹³ in which a repeat expansion mutation has been demonstrated. The expansion of the dodecamer repeat in EPM1 represents the first case of instability of a repeat unit other than trinucleotides in association with human disorders. The dodecamer contains a shorter repeat of CCCC within it, but the expansion involves the entire dodecamer and not the shorter pentamer. Other human disorders could be due to expansion of this dodecamer, or other repeats, particularly in recessive diseases showing a deficit in conventional mutations. A fragile site on human chromosome 16 was recently shown to be due to an AT-rich 33-base pair (bp) minisatellite repeat¹⁴.

The CSTB gene is ubiquitously expressed, producing a transcript of about 0.8 kb (refs. 3, 15). In order to study the results of the dodecamer expansion in the levels of CSTB mRNA, we used RNase protection assays of exon 2 of the gene in total RNA from blood leukocytes and/or lymphoblastoid or fibroblast cell lines from patients, heterozygote carriers, and normal controls (Fig. 4). We observed a marked reduction of protected CSTB mRNA in blood leukocytes from patients homozygous for the expansion compared to normals (10–20%). In contrast, in fibroblasts and lymphoblastoid cell lines, the amount of CSTB mRNA was either normal or only slightly reduced (Fig. 4). Thus, reduction of CSTB mRNA in some cell types, caused by the repeat expansions, is likely to be the mechanism of pathogenesis in EPM1. The expansions of trinucleo-

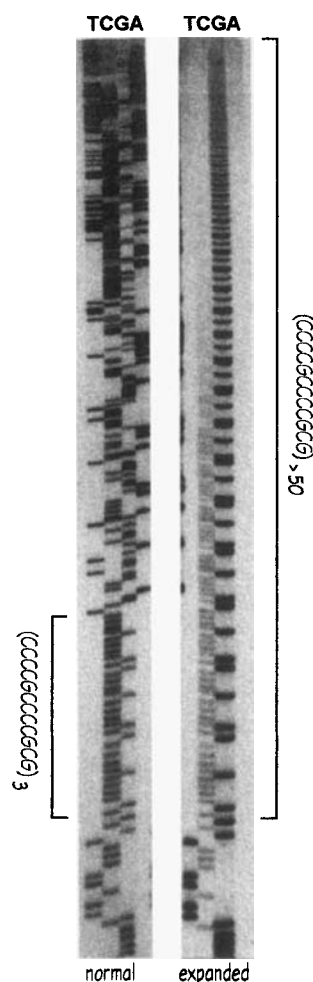


Figure 2 Nucleotide sequence analysis of a normal and a mutant clone that contain the region of the dodecamer repeat in the 5' flanking region of the CSTB gene using oligonucleotide primer 10R (see Fig. 1a). The mutant clone contains more than 50 identical, uninterrupted copies of the repeat.

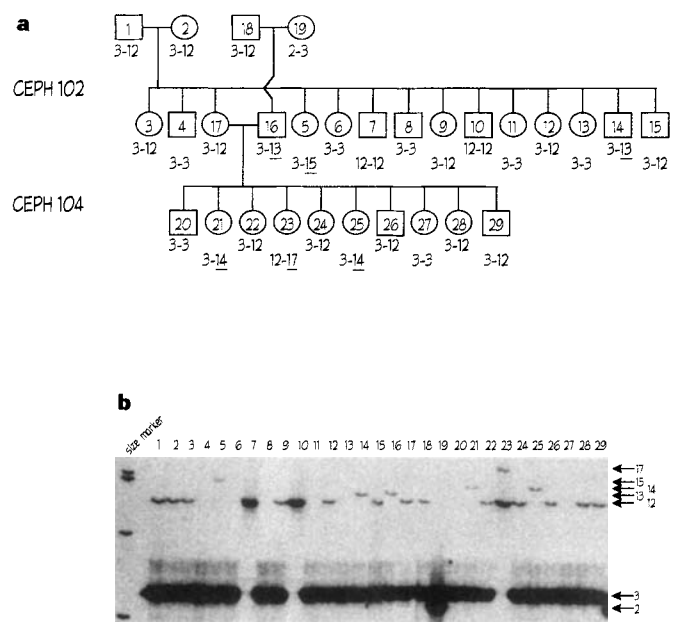


Figure 3 a, CEPH pedigrees 102 and 104 showing the number of the 12-mer repeats in the CSTB alleles. For example 3-12 refers to a genotype with 3 and 12 repeats. *De novo* alleles not present in the parents are underlined. Non-paternity has been excluded by the use of thousands of polymorphic markers in these families. **b**, Autoradiogram of genotypes of the 12-mer polymorphic repeat in families 102 and 104. The oligonucleotide primers used for the PCR were 8L and 10R (see Fig. 1a). The number of repeats are indicated on the right. The lane numbering corresponds to the numbers within the pedigree symbols.

tide repeats in both Fragile X syndromes and Friedreich's ataxia are associated with decreased levels of RNA in all tissues examined^{13,16-18}, whereas for myotonic dystrophy there exist contradictory reports of both decreased and increased expression of the gene^{19,20}.

Reduction of CSTB gene expression may be due to DNA methylation differences similar to those observed in the Fragile X syndromes with the FMR1/FRAXA²¹ and FMR2/FRAXE^{17,18,22} genes. However, Southern blot analysis with *Pst*I or *Eco*RI and *Hpa*II/*Msp*I (Fig. 1a) revealed no obvious differences in DNA methylation in blood leukocytes or lymphoblastoid cell lines between all normal, heterozygote and EPM1 samples studied (data not shown). Methylation to roughly the same levels and at the same sites was observed in all samples, but not hypermethylation as observed in the FRAXs. Thus the reduction of CSTB RNA levels in blood leukocytes is apparently not associated with methylation differences.

In four of the trinucleotide repeat expansion disorders the repeat is not within the coding region whereas in all the others it encodes polyglutamine. In FRAXA syndrome and myotonic dystrophy the repeat is in the 5' untranslated region(UTR) and 3' UTR of these genes, respectively, whereas in Friedreich's ataxia the repeat is within an intron of the gene^{13,23,24}. In FRAXE there is contradictory data as to whether the repeat is or is not within the 5' UTR of the FMR2 transcript^{17,18}. However, it is most likely that the repeat is within the

transcript (J. Gecz, personal communication). We determined the transcription start sites of CSTB by RNase protection and observed two protected fragments differing by about 11 nucleotides (Fig. 5). The main transcription start site appears to coincide with the expressed sequence tag closest to the 5' end (EST) (GenBank H81985), and that published in ref. 3. In addition, 5' RACE (rapid amplification of cDNA ends) from brain RNA did not detect any sequences any further towards the 5' end of the detected transcription start sites (data not shown). Both potential transcription start sites are on the 3' side of the repeat (about 66 or 77 nucleotides downstream), and thus the repeat is not included in these transcripts.

The low level of mRNA in blood leukocytes compared with the normal level in lymphoblastoid cell lines suggests that the repeat expansion modulates the expression of the CSTB gene in some cell types. This reduction could be due to a simple increase of the distance between DNA-binding sites of cell-specific transcription factor(s) and the transcription initiation sites²⁵. A second possibility is that the dodecamer is the binding site of transcriptional repressors and the availability of many copies of this sequence results in multiplicity of the negative regulatory effect^{26,27}. Another possibility is that in certain cells the CSTB gene uses another, unidentified, transcription start site. This could result in the inclusion of the repeat expansion within the primary CSTB transcript. Thus, in

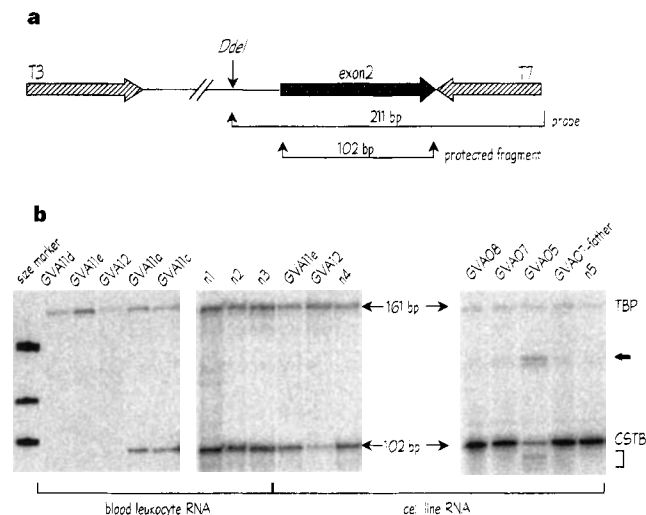


Figure 4 RNase protection assays of exon 2 of the CSTB gene. **a**, Schematic representation of the plasmid construct pEX1-2gf containing exons 1 and 2 of CSTB. The plasmid was linearized with *Dde*I digestion and a riboprobe was produced with T7 RNA polymerase. The orientation of the exon is shown by the arrow. The expected protected fragment is 102 bp. The control probe was a fragment of the TATA-binding protein (TBP) cDNA. The expected control TBP protected fragment is 161 bp. **b**, Autoradiogram of RNase protection showing the expression of CSTB relative to TBP in blood leukocytes, lymphoblastoid cell lines and cultured fibroblasts (GVA08, homozygous for the expansion); n, normals. The arrow and bracket on the right depict the alternatively spliced protected fragments from patient GVA05 who is a compound heterozygote for an expansion and an AG to AC mutation of IVS1 acceptor splice site⁴. These protected products are likely to originate from the utilization of cryptic splice sites in the allele that harbours the splicing mutation. The sizes of the protected fragments are indicated. GVA11d, e, a, and c are four siblings from family GVA11; d and e are homozygous for the repeat expansion, whereas a and c are unaffected. In blood leukocytes, there is a marked reduction of CSTB RNA in patients; in contrast, in most of the cell lines from patients its expression is not different from normal. Note that in the blood sample of GVA11e there is almost undetectable CSTB RNA, whereas in his cell line (GVA11e LBL) this RNA is within the normal limits. Patient GVA12 shows more CSTB RNA in the LBL than in blood leukocytes, however, the expression in LBL is lower than normal.

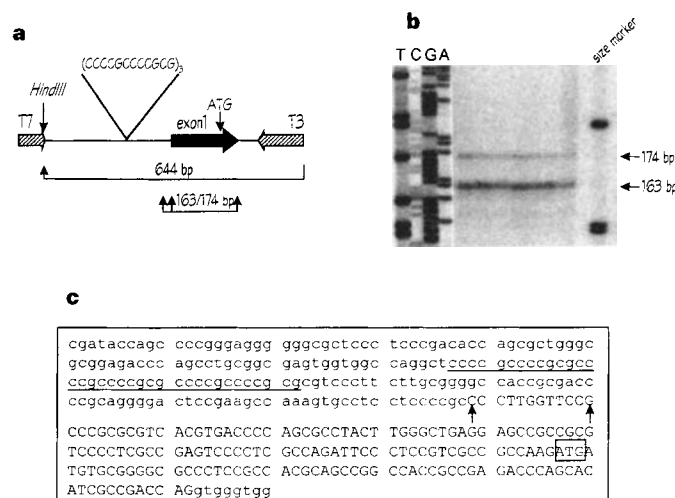


Figure 5 RNase protection assays of exon 1 and 5' flanking region of the CSTB gene. **a**, Schematic representation of the plasmid construct pEX1gr containing exon 1 and about 400 bp 5' to the ATG initiation codon of CSTB. The plasmid was linearized with *Hind*III digestion and riboprobe was produced with T3 RNA polymerase. The orientation of the exon is shown by the arrow. **b**, Autoradiogram showing two protected fragments that include the exon 1 of CSTB. Their sizes of 163 and 174 nucleotides were estimated from the sequencing ladder on the left. The predicted transcription initiation sites are distal to the dodecamer repeat as shown in **c**. **c**, Partial nucleotide sequence of the CSTB genomic region that includes the dodecamer repeat (underlined), the two predicted transcription initiation sites (arrows), and the translation initiation codon (boxed). Upper case letters denote exon 1, and lower case letters the 5' flanking region.

patients with the expansion, this could result in a reduction of CSTB RNA or a block of translation initiation.

In most disorders of trinucleotide repeat expansion, there is a correlation of early age of onset and severe disease progression with higher copy number of the repeats^{10–12}; however, this is as yet unknown for EPM1.

The fact that there are five different CSTB point mutations in all three exons^{3,4}, and that the inheritance of EPM1 is autosomal recessive, clearly implicate the absence or reduction of CSTB protein level and/or function in EPM1; however, the pathophysiology remains a mystery. CSTB is a ubiquitously expressed, cytosolic protein that functions as an inhibitor of cysteine proteases^{15,28}, some of which are involved in apoptosis²⁹. The specific cell destruction in the brains of patients with EPM1 may be due to the lack of inhibition of cell-specific proteases and accumulation of degradation products. It is also possible, however, that CSTB may have functions other than protease inhibition. The creation of a targeted disruption of the mouse *Cstb* gene³⁰ in embryonic stem cells and in mice will probably elucidate the cellular and tissue abnormalities of the absence of cystatin B.

Note added in proof: While this paper was under review, Lafrenière *et al.*⁵ have shown that EPM1 patients have various larger DNA restriction fragments of the CSTB gene. □

Methods

Patients, RNA and DNA isolation and Southern blot analysis. A description of the patient group, diagnostic criteria and clinical information can be found in ref. 4. Total RNA from blood leukocytes (prepared on Ficoll) and lymphoblastoid cells was isolated using TRIzol Reagent (Gibco-BRL). DNA was prepared using standard techniques. For Southern blot analysis, 5 µg of genomic DNA were digested with *EcoRI*, or *EcoRI* and *HindIII*, or *PstI*. For the methylation studies, DNA was digested with *PstI* or *EcoRI* and *HpaII*/*MspI*. The probe was prepared by reverse transcribed PCR on total RNA from a lymphoblastoid cell line from a normal individual using primers 1L: 5'-CGTGACCCAGCGCCTACTT-3' and 1R: 5'-GATCACAAGTGCACGCTCTG-3'. All PCRs reported in this paper were as previously described⁴, unless otherwise specified. The product was subsequently purified by QIAquick PCR purification columns and labelled by random priming. Hybridization used QuickHyb (Stratagene) for 4 h at 65°C.

Cloning and sequencing. DNA from two unrelated affected individuals (GVA05, a French patient who was compound heterozygous for the abnormal genomic fragment and a splicing mutation, and GVA08 a patient from Valais, Switzerland, homozygous for the abnormal fragment) were digested with *EcoRI* and *HindIII* and electrophoresed on agarose. Fragments between 4.5 and 7.5 kb were gel-purified using QIAEXII Gel extraction kit and cloned into the *EcoRI* and *HindIII* sites of pBluescript (Stratagene). The resulting genomic libraries were transferred to filters and screened with the probe described above. Plasmid DNA from clones containing the expansion was prepared with Qiagen columns. Sequencing was performed using Cyclist Exo *Pfu* sequencing kit (Stratagene) or ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham); for both reactions 15–20% DMSO was necessary.

Polymerase chain reactions. The PCRs shown in Fig. 1c were performed with oligonucleotide primers 11L: 5'-GAATTCCTTAAGACGTTTCAGATCCTTAATTC-3'; 11R: 5'-GAGAACTGAAGGCTGAAG-3'; R14: 5'-CCACTTTTAGCAAGAAGCCTGGCT-3'; pF4: 5'-CGCAGGGGACTCCGAAGCCAAA GTG-3'. PCR conditions were as described above. PCR amplification to determine the number of dodecamers in normal alleles was performed in a volume of 20 µl containing 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl pH 8.8, 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 1.25 mM of each dNTP (50% of dGTP was 7-deaza-2'-dGTP), 1.15 mM of each primer, 10% DMSO and 1 unit of *Taq* polymerase. End-labelled primers were used, and cycling conditions were as before⁴, except that there were 5 touch-down cycles instead of 10 and for the remaining 25 cycles the annealing temperature was 60°C. Oligonucleotide primers used are 8L: 5'-CTGCAGGATTGCCCCTACTCCGACTG-3'; and 10R: 5'-GGGGTCACGTGACGCGCGGGCGGAACCAAG-3'. After addition of loading buffer (final formamide concentration 50%) the samples were denatured and analysed on a 6% acrylamide-urea gel.

Riboprobes, RNase protection assay and 5' RACE. Plasmid pEX1-2gf containing exons 1 and 2 of the CSTB gene was linearized using *DdeI* (Fig. 4a). Plasmid pEX1gr containing exon 1 was linearized with *HindIII* (Fig. 5a), and control plasmid pTBP with *SspI*. Riboprobes were produced with MAXIscript (Ambion) and gel-purified. Total RNA (10 µg) from blood leukocytes, cultured fibroblasts or lymphoblastoid cell lines were mixed with both CSTB and TBP probes and subjected to RNase protection using HybSpeed RPA kit (Ambion). For the quantification of CSTB mRNA with the exon 2 riboprobe, a total of 21 normal blood samples and 7 normal cell lines were used. Protected fragments were electrophoresed in a 6% denaturing gel. Intensity of signals were quantified with a Molecular Dynamics Phosphorimager. 5' RACE was performed on adult human brain RNA (Clontech) using the Marathon cDNA amplification kit as recommended by the manufacturer. Following reverse-transcription, PCR was performed with oligonucleotide primers AP1 (Clontech) and 2R: 5'-TCAGTCTCTGGCTGAAGGG-3'. Products were sequenced either directly or after cloning.

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- Norio, R. & Koskineniemi, M. Progressive myoclonus epilepsy: genetic and nosological aspects with special reference to 107 Finnish patients. *Clin. Genet.* **15**, 82–398 (1979).
- Genton, P., Michelucci, R., Tassinari, C. A. & Roger, J. The Ramsay-Hunt syndrome revisited: Mediterranean myoclonus versus mitochondrial encephalomyopathy with ragged-red fibers and Baltic myoclonus. *Acta Neurol. Scand.* **81**, 8–15 (1990).
- Pennacchio, L. A. *et al.* Mutations in the gene encoding Cystatin B in progressive myoclonus epilepsy (EPM1). *Science* **271**, 1731–1734 (1996).
- Lalot, M. D. *et al.* Identification of mutations in *Cystatin B*, the gene responsible for the Unverricht-Lundborg type of progressive myoclonus epilepsy (EPM1). *Am. J. Hum. Genet.* **60**, 342–351 (1997).
- Lafrenière, R. G. *et al.* Unstable insertion in the 5' flanking region of the cystatin B gene is the most common mutation in progressive myoclonus epilepsy type 1, EPM1. *Nature Genet.* **15**, 298–302 (1997).
- Lehesiö, A.-E. *et al.* Localisation of the EPM1 gene for progressive myoclonus epilepsy on chromosome 21: linkage disequilibrium allows high resolution mapping. *Hum. Mol. Genet.* **2**, 1229–1234 (1993).
- Dausset, J. *et al.* Centre d'étude du polymorphisme humain (CEPH): collaborative genetic mapping of the human genome. *Genomics* **6**, 575–577 (1990).
- Talbot, C. C. *et al.* The tetranucleotide repeat polymorphism D21S1245 demonstrates hypermutability in germline and somatic cells. *Hum. Mol. Genet.* **4**, 1193–1199 (1995).
- Labauge, P. *et al.* Allelic heterogeneity of Mediterranean myoclonus. *Ann. Neurol.* (in press).
- Paulson, H. L. & Fischbeck, K. H. Trinucleotide repeats in neurogenetic disorders. *Annu. Rev. Neurosci.* **19**, 79–107 (1996).
- Ashley, C. T. & Warren, S. T. Trinucleotide repeat expansion and human disease. *Annu. Rev. Genet.* **29**, 703–728 (1995).
- Richards, R. I. & Sutherland, G. R. Dynamic mutations: a new class of mutations causing human disease. *Cell* **70**, 709–712 (1992).
- Campuzano, V. *et al.* Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science* **271**, 1423–1427 (1996).
- Yu, S. *et al.* Human chromosomal fragile site *FRA16B* is an amplified AI-rich minisatellite repeat. *Cell* **88**, 367–374 (1996).
- Türk, V. & Bode, W. The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett.* **285**, 213–219 (1991).
- Pieretti, M. *et al.* Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* **66**, 817–822 (1991).
- Gecz, J., Gedeon, A. K., Sutherland, G. R. & Mulley, J. C. Identification of the gene *FMR2*, associated with *FRA16B* mental retardation. *Nat. Genet.* **13**, 105–108 (1996).
- Gu, Y., Shen, Y., Gibbs, R. A. & Nelson, D. L. Identification of *FMR2*, a novel gene associated with the *FRA16B* CCG repeat and CpG island. *Nat. Genet.* **13**, 109–113 (1996).
- Fu, Y.-H. *et al.* Decreased expression of myotonic-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. *Science* **260**, 235–238 (1993).
- Sabouri, L. A. *et al.* Effect of the myotonic dystrophy (DM) mutation on mRNA levels of the DM gene. *Nature Genet.* **4**, 233–238 (1993).
- Oberle, I. *et al.* Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* **252**, 1097–1102 (1991).
- Knight, S. J. L. *et al.* Trinucleotide repeat amplification and hypermethylation of a CpG island in *FRA16B* mental retardation. *Cell* **74**, 127–134 (1993).
- Verkerk, A. J. *et al.* Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* **65**, 905–914 (1991).
- Brook, J. D. *et al.* Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* **68**, 799–808 (1992).
- Mach, B., Steimle, V., Martinez-Soria, E. & Reith, W. Regulation of MHC class II genes: lessons from a disease. *Annu. Rev. Immunol.* **14**, 301–331 (1996).
- Levine, M. & Manley, J.-L. Transcriptional repression of eukaryotic promoters. *Cell* **59**, 405–408 (1989).
- Yousoufian, H. & Lodish, H. F. Transcriptional inhibition of the murine erythropoietin receptor gene by an upstream repetitive element. *Mol. Cell. Biol.* **13**, 98–104 (1993).
- Rawlings, N. D. & Barrett, A. J. Families of cysteine peptidases. *Meth. Enzymol.* **244**, 461–486 (1994).
- Takahashi, A. & Earnshaw, W. C. ICE-related proteases in apoptosis. *Curr. Opin. Genet. Dev.* **6**, 50–55 (1996).
- Pennacchio, L. A. & Myers, R. M. Isolation and characterization of the mouse cystatin B gene. *Genome Res.* **6**, 1103–1109 (1996).

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An *achaete-scute* homologue essential for neuroendocrine differentiation in the lung

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In *Drosophila* and in vertebrates, the *achaete-scute* family of basic helix–loop–helix transcription factors plays a critical developmental role in neuronal commitment and differentiation^{1–6}. Relatively little is known, however, about the transcriptional control of neural features in cells outside a neuronal context. A minority of normal bronchial epithelial cells and many lung cancers, especially small-cell lung cancer, exhibit a neuroendocrine phenotype that may reflect a common precursor cell population^{7–11}. We show here that human *achaete-scute* homologue-1 (*hASH1*) is selectively expressed in normal fetal pulmonary neuroendocrine cells, as well as in the diverse range of lung cancers with neuroendocrine features. Strikingly, newborn mice bearing a disruption of the *ASH1* gene have no detectable pulmonary neuroendocrine cells. Depletion of this transcription factor from lung cancer cells by antisense oligonucleotides results in a significant decrease in the expression of neuroendocrine markers. Thus, a homologue of *Drosophila* neural fate determination genes seems to be necessary for progression of lung epithelial cells through a neuroendocrine differentiation pathway that is a feature of small-cell lung cancer, the most lethal form of human lung cancer.

On the basis of the critical roles played by *achaete-scute* genes in different neurally associated cell types of *Drosophila* and vertebrates, we considered that *achaete-scute* homologue-1 might function in the differentiation of normal and neoplastic lung cells with neuroendocrine (NE) characteristics. We previously detected *hASH1* expression in several lung cancer cell lines of the small-cell lung cancer (SCLC) type¹². Two other lung cancer types, bronchopul-

monary carcinoid and non-small cell lung cancer with neuroendocrine features (NSCLC-NE)^{13–15}, also share a characteristic neuroendocrine phenotype. We examined a diverse, well characterized panel of human lung cancer cell lines^{16–22} and found *hASH1* transcripts in each classic SCLC (Fig. 1a, lanes 1–6), bronchopulmonary carcinoid (Fig. 1a, lanes 8 and 9) and NSCLC-NE (Fig. 1a, lanes 10–11) cell line tested. Non-NE lines including NSCLC (Fig. 1a, lanes 12, 13, 16–20) and variant SCLC lines (Fig. 1a, lanes 14, 15) lacked detectable *hASH1* messenger RNA. Thus *hASH1* expression and NE markers were concordant in all 20 tested lung cancer cell lines. In six fresh lung cancer tumours, *hASH1* expression was readily detected by immunostaining and RNA/RNA *in situ* hybridization in NE lung cancers (SCLC and carcinoid) but not in NSCLC tumours (data not shown).

Further evidence supporting a tight linkage between *hASH1* expression and the NE phenotype emerged from studies of lung cancer cells that had undergone either a spontaneous or experimentally induced loss of NE features. The variant SCLC line NCI-H417 (a subclone derived from the *hASH1*-positive classic SCLC NCI-H231 line²⁰) and NCI-H460 (a NSCLC-NE line that had lost NE features with passaging) both displayed a non-NE phenotype and absence of *hASH1* (Fig. 1a). Similarly, insertion of an activated Harvey-*ras* gene into NCI-H249 SCLC cells leads both to loss of NE features²³ and to >95% decrease in *hASH1* mRNA (Fig. 1b, lanes 21 and 22).

To determine whether *achaete-scute* homologue-1 influences the NE phenotype found in the small population of normal airway epithelial cells that most resemble SCLC, we investigated whether normal lung NE cells express this gene. In fetal mouse lung at day 13.5, focally intense *MASH1* mRNA hybridization signals were localized in the airway epithelium in a pattern typical for clusters of NE cells, termed neuroepithelial bodies (NEBs), and as solitary pulmonary NE cells (Fig. 2a). Serial sections from newborn mouse lung revealed that *MASH1* was localized in the same NEBs as the NE markers calcitonin-gene-related peptide (CGRP) (Fig. 2b, c) and synaptophysin (result not shown). Similarly, an 18-week-old specimen of human fetal lung also gave a *hASH1* mRNA *in situ* hybridization pattern typical for pulmonary NE cells (Fig. 2d).

These data suggested that *achaete-scute* homologue-1 might be instrumental for development of the pulmonary NE phenotype. In *Drosophila*, the *achaete-scute* genes are essential for the development of key neural structures, as shown by the failure of peripheral sensory organs to differentiate from neuroectoderm in flies with loss-of-function mutations of these genes^{1,2}. Similarly, *MASH1* is essential for normal development of multiple peripheral neural structures. The mutant transgenic mouse strain *MASH1Δ* has striking abnormalities in the olfactory neurons as well as in populations of

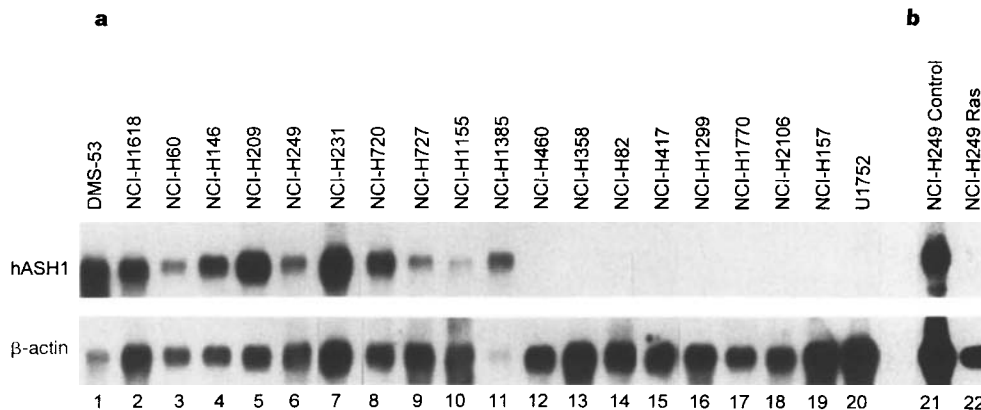


Figure 1 Northern analysis of *hASH1* expression in human lung cancer cell lines. **a**, Steady-state levels of *hASH1* mRNA in 11 lung cancer lines with neuroendocrine (NE) properties (lanes 1–11) and 9 lines without NE features (lanes 12–20). L-dopa decarboxylase (DDC) activity, a highly specific NE marker in lung cancers^{7,9}, ranged from 21 to 553 U per mg protein for lines 1–11 and was undetectable for

lines 12–20. Lines 1–7 are classified as classic SCLC; 8 and 9, bronchial carcinoid; 10 and 11, NSCLC-NE; 12, 13 and 16–20, NSCLC; and 14, 15, variant SCLC. **b**, Steady-state *hASH1* mRNA levels in NCI-H249 SCLC cells induced to a NSCLC-like phenotype following Harvey *ras* murine sarcoma virus infection²³ (lane 21) versus mock-infected control cells (lane 22).

autonomic ganglion cells, enteric neurons and adrenal chromaffin cells; death occurs within 12 to 24 hours of birth⁶.

We now find that homozygous disruption of the MASH1 gene prevents normal development of pulmonary NE cells. None of eight newborn MASH1Δ homozygotes examined had any detectable NEBs or isolated pulmonary NE cells, as characterized by a panel of NE markers (reviewed in refs 10, 13, 15), including CGRP, synaptophysin, neuron-specific enolase and chromogranin (Fig. 3b and f). In contrast, comparable sections of lungs from seven healthy newborn heterozygous mice and from five normal littermates contained over 500 NEBs or solitary NE cells, identified by staining for chromogranin (Fig. 3a), CGRP (Fig. 3e), synaptophysin or neuron-specific enolase (data summarized in Fig. 3i).

Several observations illustrate the specific role of achaete-scute homologue-1 in lung NE differentiation. First, other bronchopulmonary cell types, including Clara cells and type II pneumocytes, appeared normal by haematoxylin–eosin staining and immunostaining for multiple epithelial markers (data not shown). Second, synaptophysin and CGRP-reactive autonomic neurons persisted in the lungs of mutant mice (Fig. 3f, insert). Third, enteric neurons and NE cells in the gut and pancreatic islets from mutant mice were

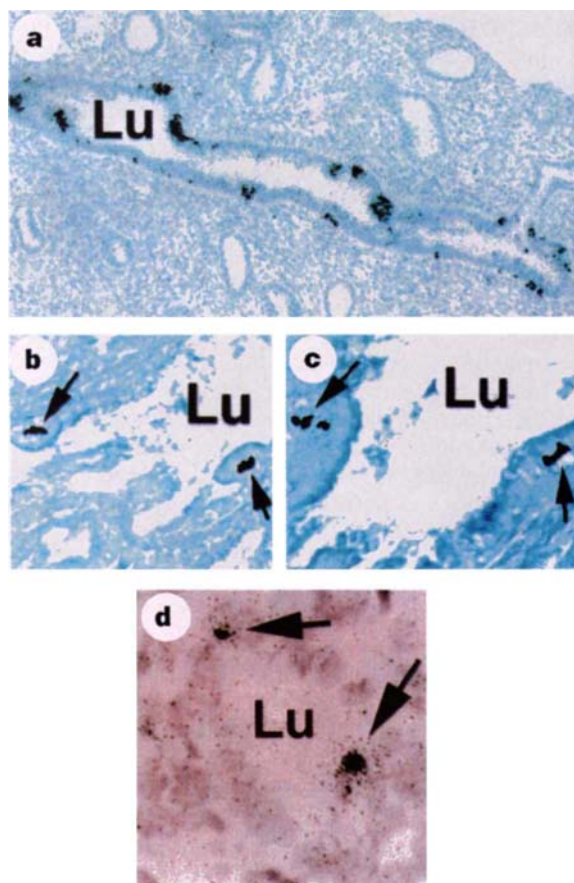


Figure 2 Photomicrographs of *achaete-scute* homologue-1 expression in fetal lungs. **a**, MASH1 RNA/RNA *in situ* hybridization of a sagittal lung section from a 13.5-day mouse embryo, with strong labelling (dense black grains) in multiple NEBs and solitary NE cells adjacent to the airway lumen (Lu). Note the two MASH1-reactive NEBs associated with the branching airway on the right. No labelling was seen in the other epithelial cells lining the airway lumen (original magnification 200 ×). **b**, MASH1 immunoreactivity in newborn mouse lung, with localization to NEBs lining an airway lumen (arrows) (immunoperoxidase; original magnification, 175 ×). **c**, CGRP immunoreactivity in a section adjacent (20 μm) to **b**, revealing expression in the same NEBs (original magnification, 240 ×). **d**, hASH1 *in situ* hybridization of a human fetal lung frozen section, with arrows indicating two hASH1-positive NE cell clusters (original magnification, 500 ×).

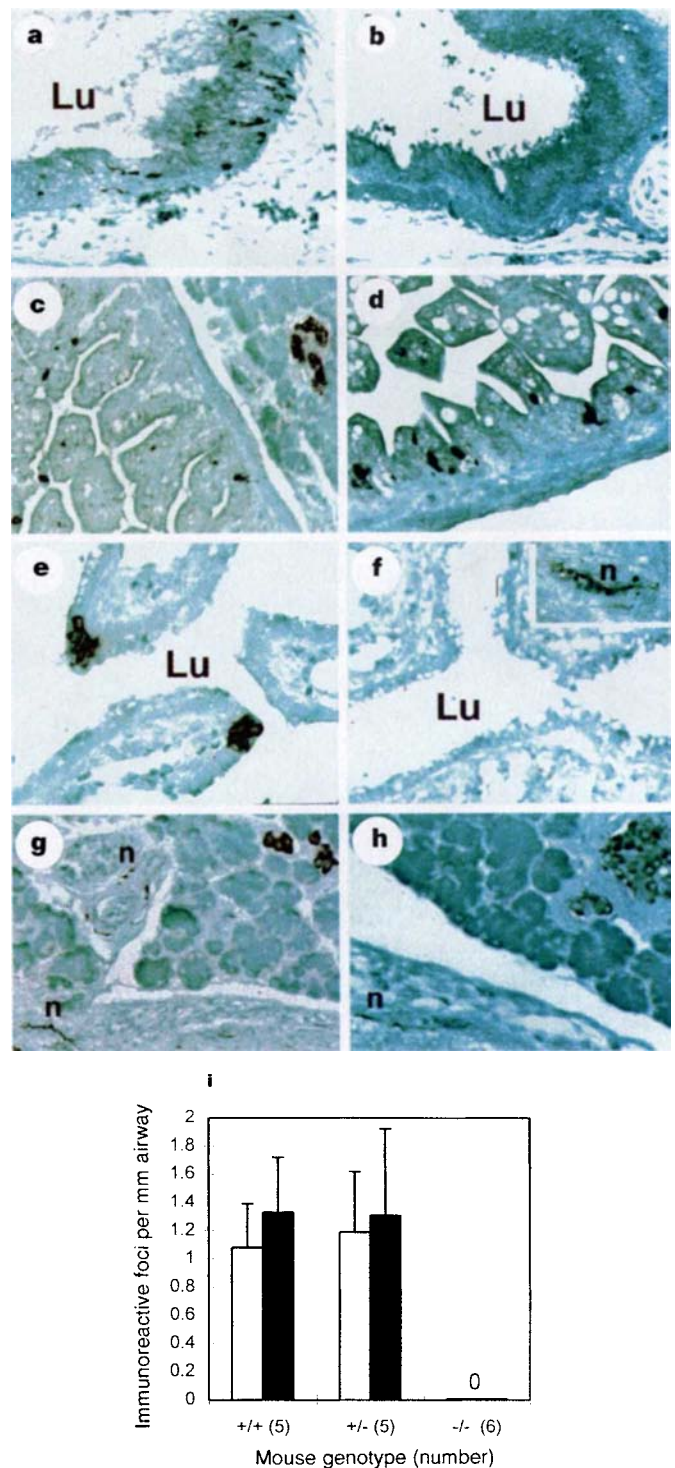


Figure 3 NE cell distribution in newborn normal (**a**, **c**, **e**, **g**) and MASH1Δ transgenic knockout mice (**b**, **d**, **f**, **h**). In the lung, normal chromogranin (**a**; original magnification, 360 ×) and CGRP (**e**; original magnification, 270 ×) immunoreactivity in solitary airway NE cells and in NEBs, clustering at airway branch points, was absent in corresponding sections from MASH1Δ mice (**b**, **f**). Lung CGRP reactivity in MASH1Δ mice was confined to scattered, beaded nerve fibres (inset (**n**)). Lu, airway lumen. In the gut, normal islet, intestinal NE cell and intestinal nerve staining for chromogranin (**c**; original magnification, 110 ×) and CGRP (**g**; original, 180 ×) appeared unchanged in MASH1Δ mice (**d**, **h**). **i**, Quantification of synaptophysin (white bars) and CGRP (black bars) reactive NEBs and solitary NE cells, expressed per mm of airway epithelium. Normal (+/+) and heterozygous (+/-) animals were similar (Wilcoxon test), whereas homozygous mutants (-/-) completely lacked these foci. Similar results were obtained when foci were normalized to airway number rather than length.

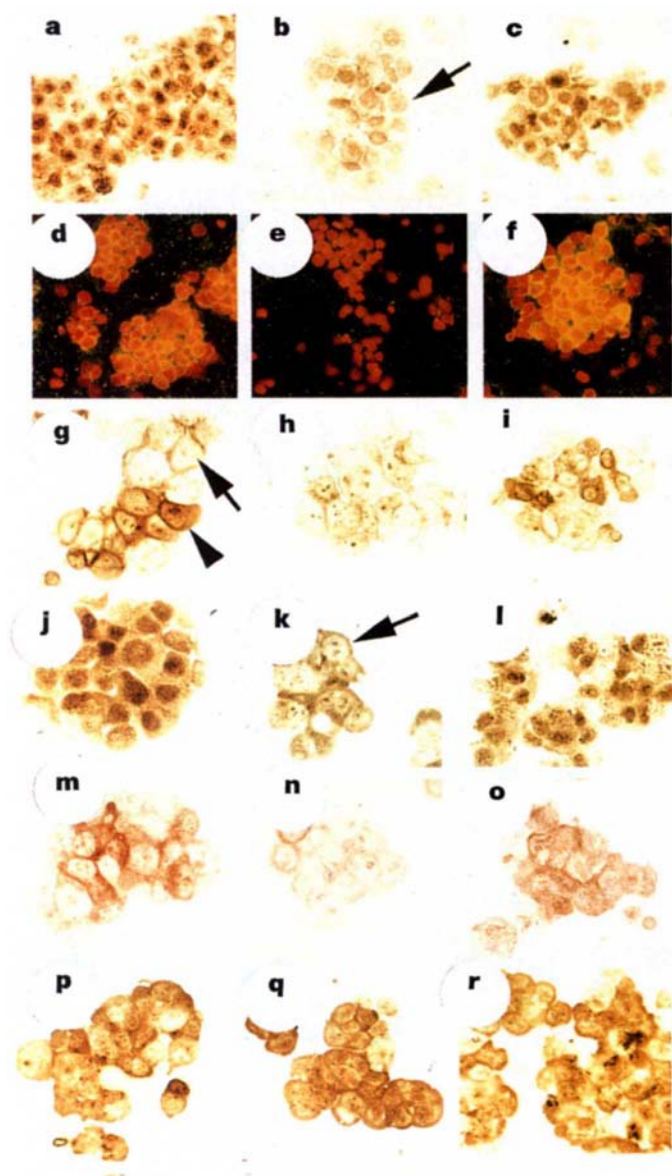


Figure 4 Depletion of hASH1 from cultured SCLC cells by antisense oligonucleotides. **a**, hASH1 immunostaining of NCI-H209 cells treated with a missense control oligonucleotide, c5pMiss1; **b**, a potent antisense oligonucleotide, c5p1476; or **c**, a minimally effective oligonucleotide, c5p25 (immunoperoxidase; original magnification, 200 $\times$). **d**, NSE staining (yellow cytoplasmic immunofluorescence with red propidium iodide nuclear counterstain) of cells treated with c5pMiss1; **e**, c5p1476; or **f**, c5p25 (immunofluorescence; 120 $\times$). **g**, Synaptophysin staining in cells treated with c5pMiss1; **h**, c5p1476; or **i**, c5p25 (immunoperoxidase; 300 $\times$). DMS53 cells treated with c5p1476 exhibited a similar reduction of hASH1 (**k**), synaptophysin (**n**) and NSE (not shown), compared to missense (**j**, **m**) and ineffective oligonucleotide controls (**l**, **o**). Calcitonin, an abundant endocrine marker in this line, was not diminished by c5p1476 (**q**), compared to missense (**p**) or ineffective antisense (**r**) controls (250 $\times$). Arrows in **b** and **k** indicate typical marked depletion of hASH1 by c5p1476 in individual nuclei. Arrows in **g** indicate the range of normal cytoplasmic synaptophysin staining. A second missense oligonucleotide, c5pMiss2, had similar effects to c5pMiss1. The oligonucleotides had no short-term effects on NCI-H209 cell survival and modest, equivalent effects on DMS53 survival. Compared to c5pMiss1, c5p1476 treatment resulted in significant reductions of hASH1, NSE and synaptophysin ($53 \pm 6\%$, $64 \pm 14\%$ and $66 \pm 4\%$, respectively; $P < 0.0001$), by quantitative morphometry. Similar results were obtained when the synaptophysin reduction was analysed by immunoblotting (not shown).

positive for chromogranin (Fig. 3c, d) and CGRP (Fig. 3e, h), as well as for other NE markers including synaptophysin, gastrin-releasing peptide and tyrosine hydroxylase (data not shown). Thus, these results favour a distinct role for achaete-scute homologue-1 in pulmonary NE cell development, rather than in the development of other NE tissues that include the endocrine pancreas and gut.

To test whether hASH1 helps to maintain the NE phenotype of established lung cancer cells, we depleted two classic SCLC cell lines of hASH1 protein by a 48-hour exposure to hASH1-specific antisense oligonucleotides. Of six tested antisense oligonucleotides, one (c5p1476) resulted in a substantial loss of hASH1 immunostaining in both NCI-H209 and DMS53 cells (Fig. 4b, k) compared to control cells treated with a missense oligonucleotide (Fig. 4a, j) or with several oligonucleotides directed at other portions of hASH1 mRNA. Depletion of hASH1 was associated with an equally dramatic downregulation of the neural markers neuron-specific enolase (Fig. 4e) and synaptophysin (Fig. 4h, n) relative to missense control cells (Fig. 4d, g, m). Interestingly, the endocrine marker calcitonin was not affected by hASH1 depletion (Fig. 4q) compared with missense (Fig. 4p). Together, these data indicate that hASH1 may be essential for maintaining the full NE phenotype of established lung cancer cells. In addition, the persistence of calcitonin expression, despite hASH1 depletion over 48 hours, suggests that at least some NE features in lung cancer can be maintained through hASH1-independent pathways and argues against a nonspecific toxic effect of the c5p1476 oligonucleotide at this time point.

We have now provided evidence for a functional link between a family of transcription factors essential for metazoan neurogenesis and the NE cells of the lung. In a model of lung cancer development¹¹, SCLC is thought to derive from transformation of lung epithelial precursor cells with commitment to a pathway of NE cell differentiation. Modulation of hASH1 may contribute to the plasticity of lung cancer phenotypes and the observed transitions of SCLC to NSCLC. The remarkable conservation of neurogenic pathways from *Drosophila* to mammals implies that regulation of hASH1 in developing lung and in lung cancer may involve inhibition through the interaction of vertebrate homologues of *Drosophila* neurogenic factors such as Notch and Hair^{24–26} as well as evolutionarily conserved positive regulators²⁷. The elucidation of these pathways should provide insight into the mechanisms underlying the determination of cell fate in the renewing bronchial epithelium and in lung carcinogenesis. □

Methods

Cell culture and RNA procedures. Cell culture^{16–22}, *ras* infection²³ and northern analysis^{12,28} have been described. Filters containing 2 μ g poly(A)⁺ RNA per lane were probed with a random-labelled hASH1 cDNA fragment (base pairs 213 to 1,635)¹², followed by autoradiography for 16 h at -70°C .

In situ hybridization and immunohistochemistry. Whole-mount mouse embryo sections hybridized with ³⁵S-labelled MASH1 RNA probe were provided by A. Joyner. RNA–RNA *in situ* hybridization with a hASH1 probe (base pairs 1,350 to 1,635) were performed on tumours as described²⁹. A polyclonal rabbit antiserum raised against a full-length glutathione-S-transferase (GST)–hASH1 fusion protein was found to immunoprecipitate *in vitro* translated and native hASH1 from SCLC cell lines and to cross-react with the murine protein (H.V.D.V. and D.W.B., unpublished data). hASH1 immunohistochemistry used a 1:1,000 antiserum dilution with an avidin-biotinylated peroxidase technique¹³. Additional immunostaining for CGRP (Amersham), neuron-specific enolase (Dako), chromogranin A (Boehringer-Mannheim), synaptophysin (Zymed), CC10 (from G. Singh), surfactant protein A (from S. Bhattacharya) and keratin (Dako) was done as described^{10,13}. A frozen specimen of fetal lung at 18 weeks gestation was obtained from autopsy of a stillborn at Johns Hopkins Hospital.

Transgenic mouse procedures. Genotyping of the MASH1Δ transgenic strain has been described⁹. Anaesthetized newborn mice were fixed in 10% buffered formalin, bisected sagittally and embedded in paraffin. 5-micron serial sections (a minimum of 2 sections 60 microns apart for each marker) were

Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration

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An increase in the intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) controls a diverse range of cell functions, including adhesion, motility, gene expression and proliferation^{1,2}. Calcium signalling patterns can occur as single transients, repetitive oscillations or sustained plateaux^{2,3}, but it is not known whether these patterns are responsible for encoding the specificity of cellular responses. We report here that the amplitude and duration of calcium signals in B lymphocytes controls differential activation of the pro-inflammatory transcriptional regulators NF- κ B, c-Jun N-terminal kinase (JNK) and NFAT. NF- κ B and JNK are selectively activated by a large transient $[\text{Ca}^{2+}]_i$ rise, whereas NFAT is activated by a low, sustained Ca^{2+} plateau. Differential activation results from differences in the Ca^{2+} sensitivities and kinetic behaviour of the three pathways. Our results show how downstream effectors can decode information contained in the amplitude and duration of Ca^{2+} signals, revealing a mechanism by which a multifunctional second messenger such as Ca^{2+} can achieve specificity in signalling to the nucleus.

Calcium signalling is important for the growth, death, differentiation and function of immune cells^{4–6}. Several Ca^{2+} -sensitive transcriptional regulators, including NF- κ B^{7,8}, JNK^{9,10} and NFAT^{4,6,11–14}, participate in varying combinations to promote the expression of genes that underlie these responses. These genes encode haematopoietic growth factors such as the interleukins IL-2 and IL-4 and GM-CSF, and inflammatory cytokines such as IL-1, IL-6, IL-8 and tumour-necrosis factor (TNF)^{4,7,10,14}. We examined the Ca^{2+} sensitivity and response dynamics of the three transcriptional regulators in B lymphocytes from mice expressing an immunoglobulin transgene specific for the hen-egg lysozyme (HEL) antigen¹⁵. Acute stimulation of B cells with HEL and phorbol ester evoked a biphasic Ca^{2+} response that consisted of an initial rapid rise of $[\text{Ca}^{2+}]_i$ from a baseline of 76 ± 3 nM to a peak of $1,367 \pm 33$ nM, which subsequently declined over 10 min to a low sustained plateau of 227 ± 5 nM (means $\pm$ s.e.m., $n = 572$ cells) (Fig. 1a). The time course of NF- κ B activation during this response was assayed by the phosphorylation and degradation of its cytoplasmic inhibitor I κ B α , or by the accumulation of the NF- κ B subunit, RelA, in the nucleus^{7,16,17}. NF- κ B activation was first detectable 4 min after stimulation with HEL and reached completion by 8 min (Fig. 1b). Like NF- κ B activation, the phosphorylation of JNK1 and its nuclear substrate ATF-2 (refs 10, 18) was also first apparent within 4 min and reached a peak by 12 min (Fig. 1c). In contrast, activation of NFATc, as determined by its translocation to the nucleus, was much more rapid, being essentially complete within 1 min of stimulation with antigen (Fig. 1d, e). The activation of each of these pathways was inhibited by the immunosuppressant cyclosporin A (CsA)¹⁹ as shown previously^{8,9,20}.

An important question is whether the spike and plateau phases of the Ca^{2+} response activate distinct transcriptional pathways. Stimulation of the antigen receptor activates Ca^{2+} -independent as well as Ca^{2+} -dependent signalling pathways^{5,21}; we therefore applied the

immunostained as described. Quantification was achieved by using Image-1 software (Universal Imaging). Positive controls included sections of adrenal medulla, thyroid, pancreas, intestine, ganglia and nerves.

Antisense studies. 100 nM phosphorothioate oligodeoxynucleotides with C-5 propyne modifications at dC and dU (Midland) were reconstituted with $2.5 \mu\text{g ml}^{-1}$ GS2888 cytofectin³⁰ (Gilead Sciences) in OptiMEM (Gibco), then incubated with 5×10^5 NCI-H209 or DMS53 cells in 6-well plates for 48 h in growth media. 5×10^4 cells were cytospun per slide. Immunostaining, quantified using MetaMorph software (Universal Imaging), included at least ten cell clusters (over 200 cells) for each marker and oligonucleotide. Experiments were done in triplicate for both cell lines and evaluated in a blind fashion. Oligonucleotides c5p1476 (5'-GGAGCCCACUGCUUU-3'), c5p1232 (5'-UCCCAACGCCACUGA-3') and c5p25 (5'-UCCUACUAGGCGUC-3') correspond to positions 1,476–1,491 and 1,232–1,247 in the hASH1 3' UTR and 25–40 in the 5' UTR, respectively; the c5pMiss1 sequence is 5'-GCUACAU-CUGGUCG-3'; c5pMiss2 5'-CACUGAUGCACCUGU-3'.

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1. Campuzano, S. *et al.* Molecular genetics of the achaete-scute gene complex of *D. melanogaster*. *Cell* **40**, 327–338 (1985).
2. Jan, Y. N. & Jan, L. Y. Genetic control of cell fate specification in *Drosophila* peripheral nervous system. *Annu. Rev. Genet.* **28**, 373–393 (1994).
3. Johnson, J. E., Birren, S. J. & Anderson, D. J. Two rat homologues of *Drosophila* achaete-scute specifically expressed in neuronal precursors. *Nature* **346**, 858–861 (1990).
4. Lo, L. C. *et al.* Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev.* **5**, 1524–1537 (1991).
5. Sommer, L. *et al.* The cellular function of MASH1 in autonomic neurogenesis. *Neuron* **15**, 1245–1258 (1995).
6. Guillemot, F. *et al.* Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463–476 (1993).
7. Baylín, S. B. & Gazdar, A. F. in *Small Cell Lung Cancer* 123–143 (Grune and Stratton, New York, 1981).
8. Gazdar, A. F. *et al.* Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. *Cancer Res.* **48**, 4078–4082 (1988).
9. Gazdar, A. F. *et al.* in *Small Cell Lung Cancer* 145–177 (Grune and Stratton, New York, 1981).
10. Linnoila, R. I. in *Neuropeptides in Respiratory Medicine* 197–224 (Dekker, New York, 1994).
11. Mabry, M. *et al.* Transitions between lung cancer phenotypes—implications for tumor progression. *Cancer Cells* **3**, 53–58 (1991).
12. Ball, D. W. *et al.* Identification of a human achaete-scute homolog highly expressed in neuroendocrine tumors. *Proc. Natl Acad. Sci. USA* **90**, 5648–5652 (1993).
13. Linnoila, R. I. *et al.* Neuroendocrine differentiation in endocrine and nonendocrine lung carcinomas. *Am. J. Clin. Pathol.* **90**, 641–652 (1988).
14. Linnoila, R. I., Piantadosi, S. & Ruckdeschel, J. C. Impact of neuroendocrine differentiation in non-small cell lung cancer. *Chest* **106**, 367S–371S (1994).
15. Linnoila, R. I. & Aisner, S. C. in *Lung Cancer* 73–95 (Wiley-Liss, New York, 1995).
16. Carney, D. N. *et al.* Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res.* **45**, 2913–2923 (1985).
17. Pettengill, O. S. *et al.* Isolation and growth characteristics of continuous cell lines from small-cell carcinoma of the lung. *Cancer Res.* **45**, 906–918 (1980).
18. Bergh, J. *et al.* Establishment and characterization of a continuous lung squamous cell carcinoma cell line (U-1752). *Int. J. Cancer* **31**, 317–322 (1981).
19. Gazdar, A. F. *et al.* Establishment of continuous, clonable cultures of small-cell carcinoma of lung which have amine precursor uptake and decarboxylation cell properties. *Cancer Res.* **40**, 3502–3507 (1980).
20. Gazdar, A. F. *et al.* Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological, and growth properties. *Cancer Res.* **45**, 2924–2930 (1985).
21. Brower, M. *et al.* Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.* **46**, 798–806 (1986).
22. Linnoila, R. I. Spectrum of neuroendocrine differentiation in lung cancer cell lines featured by cytology, markers, and their corresponding tumors. *J. Cell. Biochem.* **24** (suppl.), 92–106 (1996).
23. Falco, J. P. *et al.* v-rasH induces non-small cell phenotype, with associated growth factors and receptors, in a small cell lung cancer cell line. *J. Clin. Invest.* **85**, 1740–1745 (1990).
24. H. C. Chen *et al.* Conservation of the *Drosophila* lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. *Proc. Natl Acad. Sci. USA* (in the press).
25. Jarriault, S. *et al.* Signalling downstream of activated mammalian notch. *Nature* **377**, 355–358 (1995).
26. Ishibashi, M. *et al.* Targeted disruption of mammalian hairy and enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev.* **9**, 3136–3148 (1995).
27. Gomez-Skarmeta, J. L. *et al.* Araucan and caupolicin, two members of the novel iroquois complex, encode homeoproteins that control proneural and vein-forming genes. *Cell* **85**, 95–105 (1996).
28. Nelkin, B. D. *et al.* Transcription factor levels in medullary thyroid carcinoma cells differentiated by Harvey ras oncogene: c-jun is increased. *Biochem. Biophys. Res. Commun.* **170**, 140–146 (1990).
29. Broers, J. L. V. *et al.* Expression of c-myc in progenitor cells of the bronchopulmonary epithelium and in a large number of non-small cell lung cancers. *Am. J. Resp. Cell Mol. Biol.* **9**, 33–43 (1993).
30. Lewis, J. G. *et al.* A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl Acad. Sci. USA* **93**, 3176–3181 (1996).

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Ca^{2+} ionophore ionomycin (in the presence of phorbol ester) to identify the specific impact of each phase of the Ca^{2+} response. The Ca^{2+} spike was generated in isolation by exposing the cells to ionomycin shortly before chelating extracellular Ca^{2+} with EGTA (Fig. 2a, left). Single-cell Ca^{2+} imaging confirmed that the ionomycin-induced transient had about the same magnitude as the HEL-stimulated spike ($1,301 \pm 46$ nM versus $1,367 \pm 33$ nM, respectively; mean $\pm$ s.e.m.) and returned to baseline within 3 min. Furthermore, we verified that the Ca^{2+} spikes produced by HEL

or ionomycin are attributable to Ca^{2+} released from internal stores ($\sim 25\%$) and to Ca^{2+} influx ($\sim 75\%$) (data not shown), indicating that ionomycin and HEL mobilize the same sources of Ca^{2+} to generate the spike. As a positive control, ionomycin was applied without EGTA to create a sustained $[\text{Ca}^{2+}]_i$ increase that fully activated all three pathways (Fig. 2a, right). Stimulation with phorbol ester alone did not raise $[\text{Ca}^{2+}]_i$ (data not shown), nor did it activate NFATc, NF- κ B, or JNK1 (Fig. 1b–d, lanes marked P), confirming that all three pathways require elevated $[\text{Ca}^{2+}]_i$ for activation. In the absence of phorbol ester, Ca^{2+} ionophore alone did not activate NF- κ B or JNK (ref. 19, and data not shown); ionomycin did cause nuclear translocation of NFATc but previous studies have shown that NFAT-dependent transcription requires a second signal that can be provided by protein kinase C^4 ,¹⁴

The high- Ca^{2+} spike evoked prolonged activation of NF- κ B and JNK1/ATF-2. I κ B α became phosphorylated and was degraded within 4–8 min, and RelA accumulated in the nucleus with a parallel time course (Fig. 2b, e). The NF- κ B response was comparable when $[\text{Ca}^{2+}]_i$ was raised continuously (Fig. 2b, e). Likewise, JNK1 and ATF-2 phosphorylation reached a stable maximum 8 min after the initial increase in $[\text{Ca}^{2+}]_i$, regardless of whether the rise was transient or sustained (Fig. 2c, e). Activation of both NF- κ B and

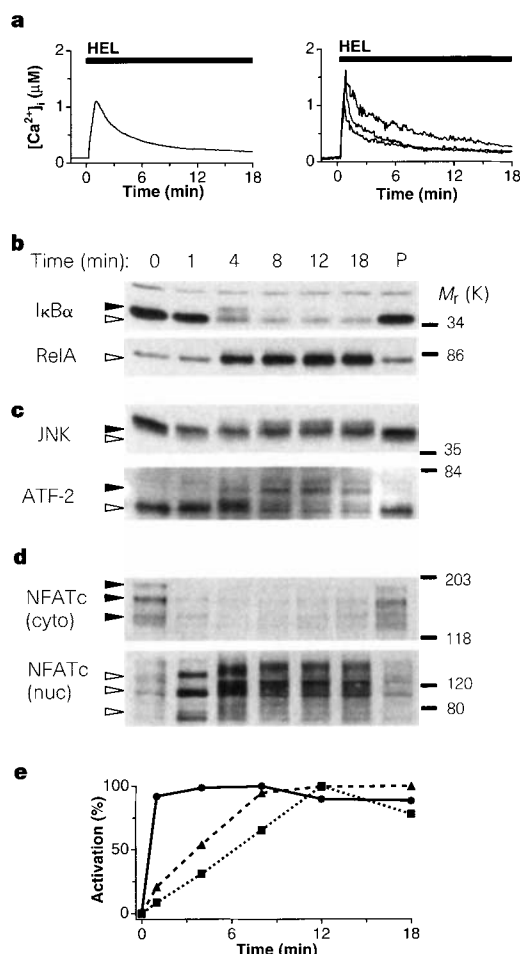


Figure 1 Antigen evokes a biphasic $[\text{Ca}^{2+}]_i$ rise that activates the NF- κ B, JNK1 and NFAT pathways. **a**, Purified splenic B lymphocytes expressing a hen egg lysozyme(HEL)-specific immunoglobulin transgene were analysed by digital video microscopy. Fura-2-loaded cells were stimulated with 500 ng ml^{-1} HEL and 5 ng ml^{-1} phorbol 12,13-dibutyrate (PDBU) (bar). Mean responses of 285 cells (left) and of representative single cells (right) are shown. After stimulation, nuclear and cytoplasmic fractions were analysed by western blotting. **b**, NF- κ B activation is demonstrated by the phosphorylation and degradation of cytoplasmic I κ B α and the accumulation of RelA in nuclear lysates. Phosphorylated I κ B α , first detectable at 4 min, has a slightly lower mobility than the dephosphorylated form. **c**, Cytoplasmic JNK1 and nuclear ATF-2 are phosphorylated following stimulation. Phosphorylation of JNK1 and ATF-2 causes a reduction in electrophoretic mobility¹⁶ that is evident at 4–18 min. **d**, NFATc translocates rapidly from the cytoplasm (cyto) to the nucleus (nuc). NFATc in its phosphorylated state is present as several bands migrating with an apparent M_r of 160K–190K (filled arrows). Nuclear NFATc is dephosphorylated and migrates more rapidly (apparent M_r 80K–150K; open arrows). In **b–d**, lane P shows lysates from cells stimulated with 5 ng ml^{-1} PDBU alone for 12 min as a control. Time in min is indicated above each lane and the M_r values (in K) are shown on the right. Filled and open arrows indicate the positions of phosphorylated and unphosphorylated species, respectively. **e**, Time course of I κ B α degradation ($\blacktriangle$), JNK1 phosphorylation ($\blacksquare$) and NFATc translocation ($\bullet$) as determined by densitometry of the immunoblots in **b–d**.

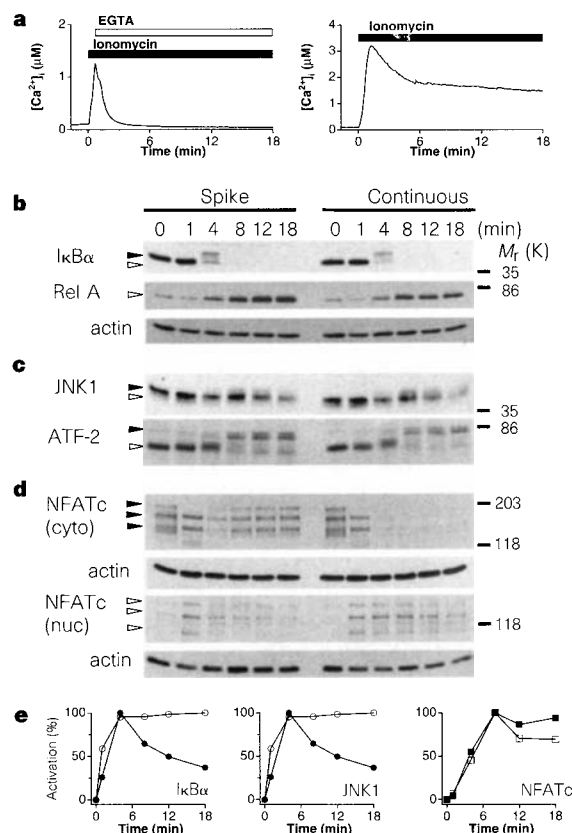


Figure 2 A brief Ca^{2+} spike induces persistent activation of NF- κ B, JNK1 and ATF-2, but transient translocation of NFATc. **a**, Average Ca^{2+} response of >250 cells treated with 950 nM ionomycin and 5 ng ml^{-1} PDBU (black bar). Left, 3 mM EGTA was added 40 s later (white bar) to terminate the spike. **b**, The Ca^{2+} spike (left lanes) and the continuous $[\text{Ca}^{2+}]_i$ rise (right lanes) activate NF- κ B similarly, as shown by I κ B α phosphorylation and degradation and RelA translocation. **c**, JNK1 and ATF-2 phosphorylation are also sustained following an isolated Ca^{2+} spike or a prolonged $[\text{Ca}^{2+}]_i$ increase. **d**, Cytoplasmic NFATc (cyto) is dephosphorylated and enters the nucleus (nuc) but returns to the cytoplasm after $[\text{Ca}^{2+}]_i$ declines to baseline (left lanes). Continuously elevated $[\text{Ca}^{2+}]_i$ maintains NFATc in the nucleus (right lanes). **e**, Time course of I κ B α degradation, JNK1 phosphorylation and NFATc translocation following the $[\text{Ca}^{2+}]_i$ spike (filled symbols) or the sustained $[\text{Ca}^{2+}]_i$ rise (open symbols). Data were from the experiment shown in **a–d**.

JNK1 was sustained for at least 16 min after termination of the $[Ca^{2+}]_i$ spike, indicating that both pathways retain a memory of transient $[Ca^{2+}]_i$ rises that outlasts the Ca^{2+} signal itself. By contrast, the Ca^{2+} spike evoked only a transient nuclear translocation of NFATc, with one half of the nuclear NFATc returning to the cytoplasm in its phosphorylated form 8 min after $[Ca^{2+}]_i$ returned to baseline (Fig. 2d, e). The reversal of nuclear translocation was

caused by termination of the Ca^{2+} spike, because nuclear NFATc persisted in cells with constant elevated $[Ca^{2+}]_i$ (Fig. 2a, d). These results confirm and extend findings showing that NFAT requires a continuous $[Ca^{2+}]_i$ rise to remain localized in the nucleus^{12,13}. They also demonstrate that differences in deactivation kinetics contribute to the differential activation of multiple transcriptional pathways by Ca^{2+} . Similar results were obtained when HEL followed by EGTA was used to generate an isolated Ca^{2+} spike (data not shown).

We next investigated whether the low- Ca^{2+} plateau triggered by antigen (Fig. 1) can evoke selective activation of transcriptional pathways. Small amounts of ionomycin were added stepwise in order to mimic the antigen-induced plateau without creating an initial spike (Fig. 3a). Low concentrations of ionomycin elicit Ca^{2+} influx in lymphocytes predominantly by activating store-operated Ca^{2+} channels that underlie the antigen-evoked $[Ca^{2+}]_i$ plateau⁶; thus ionomycin seems to mimic the Ca^{2+} -mobilizing action of antigen during the plateau as well as the spike. Stimulation of NF- κ B or JNK1 was not detectable in response to the low- Ca^{2+} plateau (Fig. 3b), whereas NFATc was activated by 70% within 12 min, suggesting that the former are less Ca^{2+} responsive than NFATc. To examine the Ca^{2+} sensitivity of each pathway in more detail, we measured the activation of NF- κ B, JNK1, NFATc and NFATp¹⁴ following a 10-min stimulation with increasing concentrations of ionomycin. Consistent with the results shown in Fig. 3, nuclear translocation of NFATc/p was induced by lower concentrations of ionomycin (79 nM; Fig. 4a) than are required to activate I κ B α degradation or JNK1/ATF-2 phosphorylation (400–600 nM; Fig. 4a). Figure 4c shows the translocation of NFATc/p, degradation of I κ B α , and phosphorylation of JNK1 and ATF-2 as a function of the peak $[Ca^{2+}]_i$ evoked by different doses of ionomycin (Fig. 4b). The results identify a range of $[Ca^{2+}]_i$ levels (200–400 nM) that promote NFATc/p translocation without activating NF- κ B or JNK1. In three experiments, the $[Ca^{2+}]_i$ needed to half-maximally translocate NFATc/p (413 ± 114 nM) was substantially lower than the level needed to attain half-maximal degradation of I κ B α (612 ± 121 nM) or phosphorylation of JNK1 and ATF-2

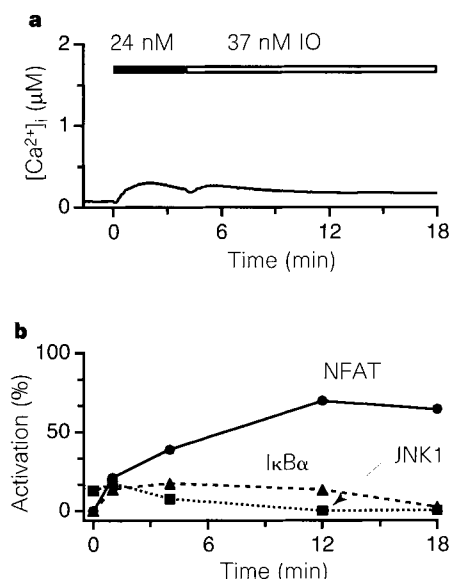


Figure 3 Selective activation of NFATc by a low $[Ca^{2+}]_i$ plateau. **a**, Cells were stimulated in the constant presence of 5 ng ml^{-1} PDBU with 24 nM ionomycin (IO) for 4 min, followed by addition of 37 nM ionomycin. The steady-state $[Ca^{2+}]_i$ plateau produced under these conditions simulates that induced by HEL stimulation (Fig. 1). **b**, Time course of NFATc translocation (●), I κ B α degradation (▲), and JNK1 phosphorylation (■) for the experiment shown in **a**.

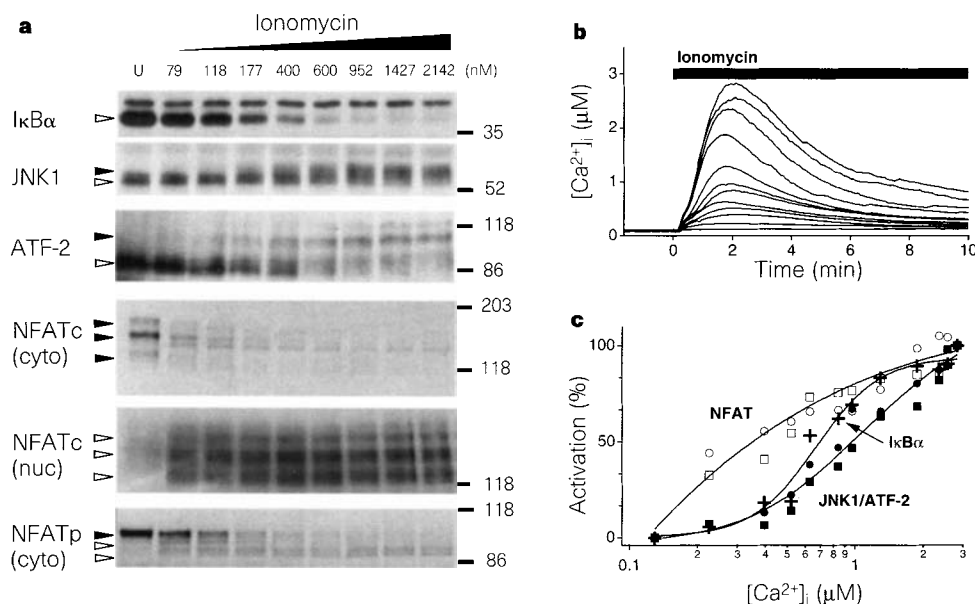


Figure 4 Differential Ca^{2+} sensitivity of NFATc/p, I κ B α /RelA and JNK1/ATF-2. **a**, Cells were treated with increasing concentrations of ionomycin in the presence of 5 ng ml^{-1} PDBU and analysed by western blotting 10 min after stimulation. The concentration of ionomycin in nM is indicated above each lane (U, unstimulated). **b**, Average $[Ca^{2+}]_i$ responses of >250 cells stimulated with increasing doses of ionomycin (23, 35, 52 nM, in addition to the concentrations shown in **a**). The same batch of cells and incubation conditions were used as in **a**. **c**, Degradation of I κ B α (+), phosphorylation of JNK1 (●) and ATF-2 (■), and translocation of NFATc (□) and

NFATp (○) are shown as a function of peak $[Ca^{2+}]_i$ for each dose of ionomycin applied in **b**. Peak $[Ca^{2+}]_i$ is most relevant for NF- κ B and JNK1 because they respond in a prolonged fashion to a transient $[Ca^{2+}]_i$ spike (Fig. 2). Because NFATc/p responds reversibly to increases in $[Ca^{2+}]_i$ (Fig. 2), this analysis may slightly underestimate the true Ca^{2+} sensitivity of NFATc/p. The graph identifies a range of $[Ca^{2+}]_i$ that selectively activates NFATc/p without appreciably stimulating NF- κ B or JNK1/ATF-2.

($1,035 \pm 263$ nM; means $\pm$ s.e.m.). These measurements of the Ca^{2+} sensitivity of NFAT translocation agree with the reported sensitivity of an NFAT/AP1-dependent reporter gene^{6,11}. The Ca^{2+} dependence of NF- κ B and JNK was previously inferred only qualitatively from their stimulation by Ca^{2+} ionophore⁹ or from inhibition by drugs like CsA that inhibit calcineurin⁸. Our results indicate that NFATc/p is significantly more Ca^{2+} -sensitive than NF- κ B and JNK1/ATF-2, and that this enhanced Ca^{2+} sensitivity enables NFAT to be selectively activated by the low- Ca^{2+} plateau.

The ability of cells to react appropriately to a wide variety of environmental stimuli requires a high degree of specificity in signalling from the plasma membrane to the nucleus. It is remarkable that such specificity is achieved, given the relatively small number of second messenger pathways that exist. Many mechanisms have been proposed to explain transcriptional selectivity²², but the contribution of different Ca^{2+} signals to specificity has not been previously recognized. Our results show that the amplitude and duration of dynamic Ca^{2+} signals contribute to transcriptional specificity, and that this is a direct consequence of the differing Ca^{2+} sensitivities and kinetic behaviour of NF- κ B, JNK and NFAT. These differences may also predict frequency-specific effects of $[\text{Ca}^{2+}]_i$ oscillations on gene transcription, which have been implicated in neuronal differentiation²³.

Differential signalling by Ca^{2+} is likely to be important in the regulation of immunity and inflammation. In self-tolerant B cells, basal $[\text{Ca}^{2+}]_i$ is moderately raised and NFAT is activated, but acute stimulation with antigen fails to generate a Ca^{2+} spike or activate the NF- κ B or JNK1 pathways¹⁹. Similarly, elevation of basal $[\text{Ca}^{2+}]_i$ and the absence of an antigen-induced Ca^{2+} spike has been reported in T cells rendered anergic by an anti-CD3 monoclonal antibody²⁴ and in T cells that have differentiated to the anti-inflammatory T_H2 state^{24,25}. Low-amplitude Ca^{2+} responses have also been implicated in the induction of T-cell anergy by altered peptide ligands²⁶. Conversely, a high Ca^{2+} spike without a lower plateau is triggered during the inhibition of B-cell responses by the binding of immune complexes to the Fc receptor^{27,28}. Our findings suggest that the functional consequences of these isolated Ca^{2+} spikes and plateaux, as well as of other Ca^{2+} signalling patterns such as oscillations and waves, may arise from the selective activation of transcriptional regulators. The importance of second messenger amplitude and duration in discriminating between different response pathways may prove to be a common theme in many cells. □

Methods

Calcium imaging. For single-cell calcium imaging, purified B cells²⁹ were incubated at 37° with $1 \mu\text{M}$ Fura-2/AM (Molecular Probes) for 15 min in phenol-red-deficient RPMI supplemented with 3% fetal bovine serum (FBS). After loading, cells were washed and maintained at room temperature in a 5% CO_2 atmosphere for up to 1 hour. Cells were plated on clean glass coverslips and imaged at 37°C in phenol-red-deficient RPMI with 1% FBS. Ratiometric imaging was done on the heated stage of a Zeiss Axiovert 35 microscope using a $40\times$ oil-immersion objective (Zeiss Achrostat, NA 1.3), an intensified CCD camera (Hamamatsu) and a Videoprobe image processor (ETM Systems) essentially as described³⁰.

Quantitation of western blots. Cells were stimulated at a density of $\sim 3 \times 10^7$ cells per ml in phenol-red-deficient RPMI with 1% FBS. Stimulations were stopped on ice, centrifuged and resuspended in hypotonic buffer (5 mM NaCl, 20 mM HEPES, pH 7.5) containing protease (2.5 mM PMSE, $40 \mu\text{g ml}^{-1}$ each of aprotinin and leupeptin, 2 mM EDTA) and phosphatase inhibitors (1 mM NaVO_4 , 6 mM pNPP, 10 mM NaF) and 0.4% NP-40. Nuclei were separated by centrifugation at $600g$ at 3°C and cytoplasmic extracts (supernatant) were added to boiling SDS-PAGE reducing sample buffer. Nuclei were rinsed in hypotonic buffer with inhibitors and lysed in boiling sample buffer. For Figs 1–3, chromatin was removed from nuclear extracts by centrifugation at $70,000g$ in a Beckman airfuge. For Fig. 4, chromatin was sheared by passing it repeatedly through a 26-gauge needle. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose and analysed by immunoblot and ECL (Amer-

sham). Anti-NFATc(7A6) and anti-NFATp(4G10G5) were obtained from L. Timmerman and G. Crabtree, anti-I κ B α and anti-RelA were from Santa Cruz Biotechnology, anti-JNK1 was from Pharmingen and Santa Cruz Biotechnology, and anti-ATF was from J. Hoefler and M. Green. Autoradiograms were analysed with a Molecular Dynamics computing densitometer using the volume-integrate mode. An exposed area of film outside each lane was used as background. Raw density values for NFATc, I κ B α , and RelA were corrected for the loading of each lane; for JNK1 and ATF-2, the density of phosphorylated bands was calculated relative to the summed density of the phosphorylated and unphosphorylated bands. NFATc activation was calculated from the loss of cytoplasmic material (measurements based on accumulation of nuclear material gave similar results). Activation is expressed relative to the values observed in unstimulated (0%) and maximally stimulated (100%) cells. On an absolute scale, maximal NFATc/p translocation ranged from 75–90%, maximal I κ B α degradation was 80–99%, nuclear RelA was induced maximally by fivefold, ATF-2 phosphorylation was 20–30%, and maximum JNK1 phosphorylation ranged from 50–60%.

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- Ghosh, A. & Greenberg, M. E. Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science* **268**, 239–247 (1995).
- Berridge, M. J. Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–325 (1993).
- Clapham, D. E. Calcium signaling. *Cell* **80**, 259–268 (1995).
- Crabtree, G. R. & Clifton, N. A. Signal transduction between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem.* **63**, 1045–1083 (1994).
- Gold, M. R. & DeFranco, A. L. Biochemistry of B lymphocyte activation. *Adv. Immunol.* **55**, 221–295 (1994).
- Fanger, C. M., Hoth, M., Crabtree, G. R. & Lewis, R. S. Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for the physiological roles of CRAC channels. *J. Cell Biol.* **131**, 655–667 (1995).
- Bauerle, P. A. & Henkel, T. Function and activation of NF- κ B in the immune system. *Annu. Rev. Immunol.* **12**, 141–179 (1994).
- Frantz, B. et al. Calcineurin acts in synergy with PMA to inactivate I κ B/MAD3, an inhibitor of NF- κ B. *EMBO J.* **13**, 861–870 (1994).
- Su, B. et al. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* **77**, 727–736 (1994).
- Karin, M. & Hunter, T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.* **5**, 747–757 (1995).
- Negulescu, P. A., Shastri, N. & Cahalan, M. D. Intracellular calcium dependence of gene expression in single T lymphocytes. *Proc. Natl. Acad. Sci. USA* **91**, 2873–2877 (1994).
- Timmerman, L. A., Clifton, N. A., Ho, S. N., Northrop, J. P. & Crabtree, G. R. Rapid shuttling of NF-AT in discrimination of Ca^{2+} signals and immunosuppression. *Nature* **383**, 837–840 (1996).
- Shibasaki, F., Price, E. R., Milan, D. & McKeon, F. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370–373 (1996).
- Rao, A. NF-ATp: a transcription factor required for the coordinate induction of several cytokine genes. *Immunol. Today* **15**, 274–281 (1994).
- Goodnow, C. C. et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682 (1988).
- DiDonato, J. A., Mercurio, F. & Karin, M. Phosphorylation of I κ B precedes but is not sufficient for its dissociation from NF- κ B. *Mol. Cell. Biol.* **15**, 1302–1311 (1995).
- Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. & Miyamoto, S. Rel/NF- κ B/I κ B family: intimate tales of association and dissociation. *Genes Dev.* **9**, 2723–2735 (1995).
- Gupta, S., Campbell, D., Derjard, B. & Davis, R. J. Transcription factor ATF-2 regulation by the JNK signal transduction pathway. *Science* **267**, 389–393 (1995).
- Healy, J. I. et al. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity* (in press).
- Flanagan, W. M., Corthess, B., Bram, R. J. & Crabtree, G. R. Nuclear association of a T-cell transcription factor blocked by FK506 and cyclosporin A. *Nature* **352**, 803–807 (1991).
- Cambier, J. C., Pleiman, C. M. & Clark, M. R. Signal transduction by the B cell antigen receptor and its coreceptors. *Annu. Rev. Immunol.* **12**, 457–486 (1994).
- Ernst, P. & Smale, S. T. Combinatorial regulation of transcription I: general aspects of transcriptional control. *Immunity* **2**, 311–319 (1995).
- Gu, X. & Spitzer, N. C. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature* **375**, 784–787 (1995).
- Gajewski, T. F., Lancki, D. W., Stack, R. & Fitch, F. W. "Anergy" of T_H0 helper T lymphocytes induces downregulation of T_H1 characteristics and a transition to a T_H2 -like phenotype. *J. Exp. Med.* **179**, 481–491 (1994).
- Gajewski, T. F., Schell, S. R. & Fitch, F. W. Evidence implicating utilization of different T cell receptor-associated signaling pathways by T_H1 and T_H2 clones. *J. Immunol.* **144**, 4110–4120 (1990).
- Sloan-Lancaster, J., Steinberg, T. H. & Allen, P. M. Selective activation of the calcium signaling pathway by altered peptide ligands. *J. Exp. Med.* **184**, 1525–1530 (1996).
- Wilson, H. A. et al. The B lymphocyte calcium response to anti-Ig is diminished by membrane immunoglobulin cross-linkage to the Fc gamma receptor. *J. Immunol.* **138**, 1712–1718 (1987).
- Choquet, D., Partiseti, M., Amigorena, S., Bonnerot, C. & Fridman, W. Cross-linking of IgG receptors inhibits membrane immunoglobulin-stimulated calcium influx in B lymphocytes. *J. Biol. Chem.* **268**, 355–363 (1993).
- Cyster, J. et al. Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. *Nature* **381**, 325–328 (1996).
- Dolmetsch, R. & Lewis, R. S. Signaling between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels generates $[\text{Ca}^{2+}]_i$ oscillations in T lymphocytes. *J. Gen. Physiol.* **103**, 365–388 (1994).

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Collected for separation

Chromatography selections for this issue include a system designed to increase the productivity of automated microbore analysis, a column for capillary electrochromatography and chromatography data system software.

Integrity system LC/MS

From Waters

A new 12-page colour brochure describing the features of its Integrity System for benchtop LC/MS

This system has been designed to be used by analysts to determine the identity and quantity of small-molecule compounds in HPLC samples quicker than previously possible. The brochure includes photographs, computer-generated illustrations and sample spectra describing the main features of the system, which includes a single quadrupole ThermoFinnigan mass spectral detector, a 'no tools' design for ease-of-use and maintenance, and single-point control. It is said to offer both electron impact mass spectra and ultraviolet spectra on a single chromatographic peak. This system should allow chromatographers, who once had to rely on a mass spectrometrist for compound identification work, to obtain the information themselves.

Reader Enquiry No. 100

7600 Series solvent D-Gassers

From Jones Chromatography

For constant on-line degassing of HPLC mobile phase

This degasser removes air/oxygen from the mobile phase without changing the solvent composition and reduces baseline drift and short-term noise to improve detection limits. The compact units eliminate the need for helium sparging and are said to provide fast and enhanced degassing stabilization time due to minimal dead volumes. The D-Gasser is available in two-, three- and four-channel models and can be used with all HPLC systems.

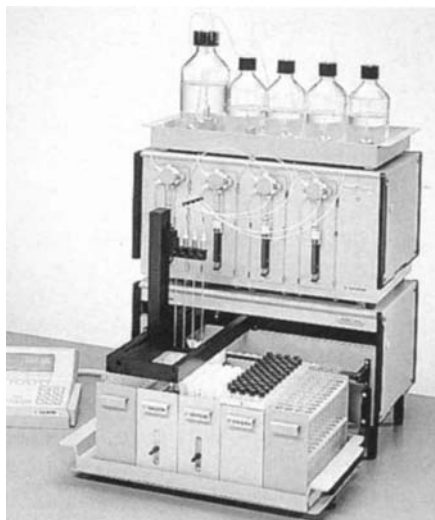
Reader Enquiry No. 101

Aspec XL4 system for SPE

From Gilson

A new, high-capacity system that automates SPE, processing up to 50 samples per hour

Designed for off-line, high-capacity solid-phase extraction (SPE), this system handles up to 108 samples in a single batch, making it useful for purification in combinatorial chemistry, LC/MS and GC. It utilizes a four-probe Cartesian arm to process four samples in parallel with identical treatment. If an SPE protocol requires about 10 min, the system can process 25 samples per hour. With a fully optimized SPE protocol, more than 50 samples can be processed in an hour. Manual protocols are easily transferred to the system. It accepts both 1 ml and 3 ml standard SPE columns and can accommo-



Gilson: parallel processing for fast throughput.

date nine solvents and four different sizes of sample vial. Both size DEC's can be used in a single run. The instrument can automatically transfer the extract to open or sealed autoinjector vials for further analysis. It can also be used for mixed-mode solid-phase extraction, multicollection and for automating sample pretreatment. Other features, which include the use of positive-pressure displacement, a liquid-level detector to minimize sample carryover, simple hydraulics and built-in, sample-needle rinsing, are designed to eliminate dead volumes, minimize cross contamination and sample loss.

Reader Enquiry No. 102

Saturn 2000 GC/MS accessories

From Varian

Three new accessories designed to enhance the performance and productivity of this system

The accessories include a new sample introduction device, ChromatoProbe; an LCI inlet for liquid chemical ionization (CI) reagents; and a CI manifold for multiple CI reagents. ChromatoProbe is said to offer analytical benefits at a lower price than that of conventional probes. An upgrade to the universal capillary injector (UCI), this unit permits qualitative spectra and even quantitative estimates on solids, liquids and slurries. Samples can be loaded directly into the ChromatoProbe and analysed with the power of GC/MS, chemical ionization MS and even MS/MS. The accessory operates inside the temperature programmable injector body, eliminating the risk of ion source contamination or performance loss. Available as either a Saturn option or upgrade, the LCI inlet offers an economical and simplified means of qualitative identifica-

tion through the use of liquid CI reagents. It is said to be well suited for drug, flavour/fragrance and environmental analyses. Once the chromatographer becomes accustomed to the power of LCI, the next step is to automate the introduction of different reagents. With the new Varian CI manifold, any of three reagents may be selected through the Saturn workstation. Alternatively, the manifold will accommodate a combination of liquids and gases. It is available as an option or can be retrofitted.

Reader Enquiry No. 103

Series 200 MS HPLC system

From Perkin-Elmer

This system is designed to increase the productivity of automated microbore analysis

An optimized microbore HPLC system for performing automated analysis with the PE Sciex API 150 MCA mass chromatographic analyser. The new system is designed to increase productivity in the analysis of proteins, oligonucleotides and polysaccharides, as well as pharmaceutical and pharmacokinetic samples. By combining microbore HPLC, automation and mass spectrometry (MS) with atmospheric pressure ionization (API), the Series 200 MS HPLC system allows the analysis of femtogram quantities of polar and labile compounds with confirmation of structures and molecular weights. The series comprises an autosampler, Series 200 micro-pump modules and the 785A ultraviolet/visible detector. The new pump provides increased mass sensitivity and reduced solvent consumption, without sacrificing precision, robustness or flexibility. There is a 225-position sample tray or the new combinatorial trays (2 × 96-well microtitre 200 µl or 1.0 ml). When combined with the PE Sciex API 150 MCA good laboratory practice (GLP) compliance is also simplified through full system control, providing complete HPLC and MS method documentation. For total analysis flexibility, users can also view ultraviolet and MS data within the same run. The Series 200 micro-pump delivers precise gradient performance with minimal delay volume. It is a combination of two pumps, providing precise gradients and pulseless flow into the mass spectrometer for microbore, narrowbore and analytical requirements. Designed for the special needs of LC/MS users, the autosampler has been optimized for microbore and LC/MS applications with its low dispersion characteristics and precision at lower injection volumes.

Reader Enquiry No. 104

CEC Hypersil C18 column

From Hypersil

The column opens the doors for chromatographers to exploit the technique of capillary electrochromatography (CEC).

Chromatograms produced from analyses using CEC Hypersil C18, when compared to those of conventional HPLC, are said to show a significant increase in both resolution and peak symmetry. Stated efficiencies of 200,000 p/m are routine, according to the company. The column can be used effectively in the laboratory to obtain high-selectivity, high-efficiency and high-speed separations of neutral molecules without micelles, allowing an easy interface to mass spectrometry. The technique of CEC, where flow is generated electrokinetically by using a high voltage, combines the advantages of high selectivity obtained with HPLC and the high efficiencies obtained with capillary electrophoresis. CEC C18 columns are available in a 50- μ m i.d. or 100- μ m i.d. capillaries in formats compatible with the Beckman P/ACE and the Hewlett Packard CEC instruments.

Reader Enquiry No. 105

HP 3D capillary electrophoresis system

From Hewlett-Packard

The company's first commercially available system capable of CEC

HP currently offers a packed capillary for CEC with C18 material, and more phases are planned. This system offers the advantages of diode-array detection without sacrificing the benefits of high sensitivity. Extended light-path capillaries, or bubble capillaries, help lower detection limits even further. A quick-change, self-aligning cartridge should allow capillary exchange in less than a minute. A line of HP capillaries, reagents and accessories extends the system's application to capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and isotachopheresis (ITP). The instrument



HP's capillary electrochromatography.

itself is controlled with the HP multitechnique ChemStation, which is based on Microsoft Windows.

Reader Enquiry No. 106

Prosep-chelating I

From Bioprocessing

A new application for separation of trace metals from an aqueous solution for analysis

This feature should be of interest to specialists in the ICP-MS and AAS fields, enabling easy preconcentration and/or matrix separation of most transition metals, lanthanides, Pb and U. Analysts gain the benefits of using this incompressible solid phase, which responds very rapidly to changes in pH, without prolonged conditioning and without shrinking or swelling. The metals of interest bind and desorb quantitatively, even at the part-per-billion level, with exceptionally good repeatability. Even awkward matrix elements like calcium can be removed by using appropriate conditions of pH. The product can be supplied loose or in prepacked columns. Prosep-chelating I is formulated to be a highly efficient adsorbent for the purification of biological molecules using the IMAC (immobilized metal affinity chromatography) technique. The matrix uses high-silica glass particles permeated by interconnecting pores of uniform and precisely controlled size. It exhibits chemical and physical stability and is inert to most acids, detergents, organic solvents and buffers of varying ionic strength. It is dense, incompressible and extremely durable. It has a narrow pore size distribution (80 percent of pores fall within 10 percent of the nominal diameter) and a large internal surface area.

Reader Enquiry No. 107

Radiomatic HPLC detector

From Packard Instrument

Utilizes scintillation counting technology that should satisfy the sensitivity requirements of analytical chemists and biochemists in drug metabolism, toxicology, environmental disposition and drug synthesis laboratories

The Radiomatic systems exhibit high performance with the company's time-resolved liquid scintillation counting TR-LSC technology, which is said to reduce background by a factor of four. An integrated multichannel analyser performs full-spectrum analysis samples. These features are stated to increase signal-to-noise by 300 percent. In addition, the 500TR and Flo-One for Windows radiochromatography software have built-in features that help analysts comply with good laboratory practice regulations, such as self-normalization calibration, instrument performance assessment and a complete documented audit trail during radio-analytical method development.

Reader Enquiry No. 108

Genesis robotic sample processors

From Tecan

Rapid, automated solid-phase extraction (SPE) is possible with robotic sample processors (RSPs)

Bringing new efficiency and convenience to a wide range of applications, SPE on this series of RSPs is suited for sample clean-up, filtration and extraction of specific molecules.

The Genesis series RSPs cater for both negative- and positive-pressure SPE. Flexibility is combined with throughput, the manufacturer states, with a choice of either four or eight pipetting probes and the capacity of the Genesis series worksurfaces. These allow up to 240 samples to be extracted in a single run. Moreover, controlled pressure is applied by the pipetting probes for full automation of positive-pressure SPE. Negative-pressure SPE is also fully automated using Porvair filters, and minimal manual intervention is required for other filter types. The 96-tube format of Porvair filters is also compatible with the series' robotic manipulator arm for extended automation capabilities. The system is controlled through dedicated Windows software packages for both positive and negative pressure. The ability to link the software with other liquid handling packages allows integration of SPE with other series capabilities for extended automation of even the most complex protocols. The three instruments in this series are designed to meet the liquid handling demands of all workloads. All can accommodate any combination of Teflon-coated stainless steel tips and disposable tips (200 μ l and 1,000 μ l), with advanced liquid-level detection operating with both tip types.

Reader Enquiry No. 109

PL Caliber GPC/SEC software

From Polymer Laboratories

Version 7.0 runs under Windows 3.1, 95 and Windows NT Workstation 4.0

Windows NT compatibility ensures powerful GPC software from PL, which runs under a networking, multiuser operating system with the benefits of strong security. It also supports AIA format data for easy collection and reanalysis of data from other manufacturers' systems. This new version of this software has the advantage of user interface enhancements and features that include new peak and baseline detection options, analysis of multiple peaks, new integration marker options, negative peak detection for HTGPC, automatic reporting of plate counts, and additional electronic reporting options.

Reader Enquiry No. 110

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.